THE EFFECTS OF A VITAMIN B_{12} SUPPLEMENT, VITAMIN B_{12} AND STREPTOMYCIN ON THE METABOLISM OF THE RAT [1]

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Much attention has been focused on recent reports in which crude vitamin B_{12} (animal protein factor) supplements, as well as pure vitamin B_{12} and antibiotics, have been included in rations for chickens and swine, the claim being made, as a result of this work, that the animals gained weight more rapidly and usually more efficiently than did other animals consuming comparable rations without these supplements (Stokstad and Jukes, '50; Stevens, Biely and Marsh, '49; Jukes, Stokstad, Taylor, Cunha, Edwards and Meadows, '50). Most of the rations used in this work employed a few basic feeds, plus vitamin and mineral supplements, with the protein being supplied largely from plant sources such as grains and soybeans, but in certain cases the effects were also obtained with rations containing animal protein. Of primary interest to the authors was the metabolism of energy and its possible correlation with the increased efficiency of utilization of rations containing vitamin B_{12} and antibiotics.

The present work was undertaken to study mainly the manner in which the energy of such rations was utilized for body gain and the amount which was lost as heat production and in the excreta. Along with the study of energy utilization, it was planned also to follow the metabolism of nitrogen. The study of energy metabolism was, however, of greatest inter-

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est, since there were no other reports in the literature dealing with this subject except in a broad sense, in that such factors as body growth and feed intake only had been considered. A review of the literature on the effects of APF supplements and antibiotics on growth is therefore considered unessential for this report.

In the present work albino rats were selected as the experimental animals because of the technique involved in determination of energy balances. Although most of the previous work on this subject has been done with either chickens or swine, the method of experimentation and the equipment required to work with these types of animals would have made the project prohibitive.

The present report concerns two separate experiments, the object of the first experiment being to feed to rats a commercial vitamin B_{12} supplement,[2] containing an antibiotic, in an all-plant protein ration. The second experiment was undertaken after completion of the first to study the response of rats when given the main component parts, in relatively pure form, of the same vitamin B_{12} supplement that was used in the first experiment. The components of the vitamin B_{12} supplement investigated were vitamin B_{12}, free of antibiotic, and streptomycin, free of vitamin B_{12}. These were fed separately and in combination, but always in amounts equivalent to those used in crude form in the first experiment.

EXPERIMENTAL

First experiment

Three litter-mate groups of weanling albino rats, each of 11 or 12 animals, were used in this experiment. One of the groups was fed the supplemented ration, with feed consumption on an ad libitum basis. The two other groups were pair-fed, one being given the supplemented ration, the other the basal ration with no supplementation. The composition of the basal ration as given in table 1 was patterned after that

[2] Bi-Con APF — 3 +.

of rations which had been used for similar work with chickens and swine. In order to have a ration low in vitamin B_{12}, protein-containing feedstuffs of plant origin only were used. Vitamins and minerals were added in quantities considered ample for optimum growth.

The amount of vitamin B_{12} supplement added to the ration was somewhat greater than that recommended by the manufacturer at the time as supporting the best growth effects

TABLE 1

Composition of rations[1]

INGREDIENT	AMOUNT
	%
Soybean oil meal (solvent-ext.)	30.00
Linseed oil meal (solvent-ext.)	12.24
Salt mixture IV[2]	4.00
Corn oil	5.00
Dextrin	24.38
Cerelose	24.38

[1] To the supplemented ration was added 0.5% of a commercial APF supplement known as Bi-Con APF-3 +. This material, as well as the vitamin B_{12} and streptomycin, was generously supplied by Chas. Pfizer and Co., Inc., Brooklyn, N. Y.

Vitamin supplements were added per kilogram of ration as follows: Oleum percomorphum 570 mg, tocopherol (mixed) 150 mg, choline 4.00 gm, inositol 2 gm, folic acid 25 μg, biotin 10 μg, thiamine HCl 5 mg, riboflavin 5 mg, pyridoxine HCl 6.25 mg, niacin 6.25 mg, calcium pantothenate 50 mg, para-aminobenzoic acid 95 mg, and 2-methyl-1,4-naphthoquinone 2.5 mg.

[2] Lichstein et al. ('46).

when fed to chickens and swine. This supplied to the ration 33 μg of vitamin B_{12} activity per kilogram of feed and approximately 5.5 mg or 5,500 units of streptomycin per kilogram.

Feed consumption was measured in all three groups. The duration of the experiments was determined in a more or less arbitrary manner which was based on the average final weight attained by the ad libitum-fed rats during 5 weeks. For the males this was 262 gm and for the females it was 181. These weights were considered to be representative of

the period of most rapid growth of the rat and were used as a true measure of the effect of the ration on growth.

For the two groups of animals on the paired feeding regimen, the experimental procedure designated the body balance method was used (Swift, Kahlenberg, Voris and Forbes, '34). In this method body gains of energy and of protein are determined by the difference between values for these constituents determined with a control group selected and sacrificed, to represent the experimental subject at the start of the investigation, and corresponding values for the animals killed at the end of the experiment. The heat production was measured as the gross energy of the feed minus the energy of the excreta and of the body gain for the entire experimental period.

Results from the ad libitum-fed group are concerned mainly with feed consumption and gain in body weight (table 2), as excreta were not collected from this group. The information on feed consumption and gain is presented for males and females, both separately and combined. The efficiency of utilization of the ration or body gain per unit of feed consumed is remarkably high for the males, the difference in this value for the two sexes being largely a matter of the normal difference in growth characteristics of the sexes. Also, for this reason, standard errors are not reported in the first experiment since a difference of considerable magnitude is to be expected when comparing rats of both sexes, and consequently a large standard error would result. To obtain the significance of differences in results between the groups of pair-fed animals, the method of "Student" was used to compute odds.

The contrast between the efficiency of utilization of the ration by the ad libitum-fed rats and the paired group receiving the basal ration alone is striking. As practically all the refusals of feed in the pair-fed groups occurred in the control or unsupplemented group, it may be assumed that this group was essentially fed on an ad libitum basis. The only difference in the rations was the vitamin B_{12} supplement,

TABLE 2

Food eaten; gains in live weight, nitrogen and fat; and efficiency of gains

RATION	DAYS ON EXP.	FOOD EATEN	INITIAL LIVE WEIGHT	FINAL LIVE WEIGHT	GAIN IN EMPTY BODY WEIGHT	NITROGEN OF BODY GAIN	FAT GAINED	FOOD/ GAIN
		gm	*gm*	*gm*	*gm*	*gm*	*gm*	
Pair-fed groups								
Control	69	754	48	217	163	5.5	15.42	4.46
Supplemented	69	754	47	235	182	6.1	24.07	4.01
Ad libitum-fed group								
Males	35	545	46	262				2.52
Females	35	417	47	180				3.14
Ave., males and females		492	46	228				2.70

the main constituents of which were presumably vitamin B_{12} and, in this case, streptomycin. It is recognized that other B vitamins, closely associated with B_{12}, might also have been present, but indications are that vitamin B_{12} was by far the most effective of these vitamins in promoting growth. Consequently the favorable results obtained with the supplement are attributed, to a very great extent, to its content of vitamin B_{12} or streptomycin, or both.

It should be pointed out that the rats receiving the supplemented ration ad libitum were consuming feed far in excess of their maintenance requirement and, therefore, a greater percentage of it was available for body gain.

Termination of the experiment for the pair-fed animals, based upon attainment of the live weight previously designated, required an average of 76 days for the males, with two animals not reaching this limit, and 64 days for the females (only 35 days were required when the feed consumption was not restricted on the supplemented ration). Obviously, there was a drastic limitation of food consumption with the pair-fed animals, the limiting being done by the animals on the unsupplemented ration. This group had 393 refusals of feed, while the supplemented group had only 25 refusals.

Further information in table 2 concerning the pair-fed groups shows an appreciable increase in empty body weight in the supplemented group as compared with the control group, amounting to over 11%. A small difference is noted in nitrogen gained which is in close relationship to the gain in body weight. This is interpreted as such and as being without fundamental significance. Evidence in table 3 tends to bear out this conclusion. In considering the rapid gain made by the rats on the ad libitum food intake, it is clear that a unit of protein was gained in this case on less feed intake than in the pair-fed group, but this was also the case with the gain of fat and the total gain in live weight. Therefore it is difficult to say which body component, in particular, was associated directly with the vitamin B_{12} supplement.

From work with rats involving a wide range of protein intake, Hove and Hardin ('51) and Hartman, Dryden and Cary ('49) concluded that vitamin B_{12} plays an important role in the utilization of protein, or in affecting the capacity of the animal to utilize protein. The present work is in complete agreement with these observations as far as growth is concerned, as it is clear that vitamin B_{12} is required for rapid growth of the rat, but in spite of a careful study of nitrogen metabolism, evidence is lacking in the present work to show that this factor is more directly associated with vitamin B_{12} than is over-all growth.

In reference again to the pair-fed groups in table 2, the amount of fat gained was much greater with the supplemented animals, indicating that not only was their gain in live weight greater but that it was also of a higher energy content.

Although not given in the tables, the average water content of the rat bodies on the control ration was 65.6% and on the supplemented ration 63.0%. These observations are in agreement with results of Ling and Chow ('51), who found that rats deficient in vitamin B_{12} had a lower body fat and higher water content than their litter mates on the same diet but receiving vitamin B_{12}. Perhaps there is a species difference in regard to fat metabolism, as there are no known reports of increased fat deposition in swine when a vitamin B_{12} supplement is fed.

It is of interest to note that this type of experiment permits a check to be made on the amount of nitrogen in the feed which is recovered in the feces and urine and as body gain. The average recovery for all the rats, except two, was 99.7%, with a coefficient of variation of 1.5.

The reason for excluding two rats from this average was a poor collection of urine. These two rats urinated while hanging to the sides of the cage, and consequently some of the urine did not fall into the crystallizing dish under the cage for collection in the normal manner. The data on these rats are excluded from all the values in table 3 involving nitrogen utilization.

The partition of energy into its final categories is in sharp contrast with the information on distribution of nitrogen (table 4), in that three important phases of energy utilization are affected by the supplement containing vitamin B_{12} and the antibiotic. The first of these noted in table 4 is the increase in metabolizable energy with the supplemented group of animals over the amount in the control group. While this difference is certainly not of great magnitude, the individual values comprising the average show very consistent differences, and odds of 400 to one are indicated.

The greatest difference found in table 4 is in the body gain of energy, which is the result mainly of the larger gain of fat

TABLE 3

Distribution of food nitrogen as affected by vitamin B_{12} supplement

DIET	CONTROL	SUPPLEMENTED
Nitrogen:		
Food, gm	21.8	21.8
Feces, gm	3.6	3.4
Digested, gm	18.2	18.4
% of food N	83.5	84.4
Urine, gm	12.5	12.5
% of food N	57.3	57.3

by the supplemented animals. From a statistical standpoint this difference is obviously highly significant. The third difference observed in the utilization and distribution of energy is found in heat production, which was greater in the unsupplemented or control group than in the group receiving vitamin B_{12} and the antibiotic. This difference is highly significant, as shown by odds of 3,300 to one.

Although not shown in table 4, the nitrogen-energy ratio in the urine of the two groups of rats was found to differ significantly. For the unsupplemented group this ration was 8.6 Cal. per gram of nitrogen in the urine, and for the supplemented group it was 7.3 Cal., the differences being very consistent in the individual comparisons making up the average.

The reasons for all the changes observed in this experiment are not evident, as the experiment was designed primarily to study effect rather than cause. Some of the more interesting of these effects are the gain of fat, the consumption of feed and the nitrogen-energy ratio in the urine. It is very significant to note the greatly increased consumption of feed when the vitamin B_{12} supplement was added to the basal diet. This change in itself is sufficient to account for perhaps all the increased gains, or efficiency of gains, made on the supplemented ration. Although many investigations have been concerned with the effect of B_{12} supplements

TABLE 4

Distribution of total energy as affected by vitamin B_{12} supplement

	CONTROL	SUPPLEMENTED
Energy value in Calories		
Food	3,021	3,021
Feces	353	348
Digested	2,668	2,673
Urine	107	87
Metabolized	2,561	2,586
Body gain — total	342	442
as protein	199	218
as fat	143	224
Heat production	2,219	2,144

on growth, there is yet little positive information on the manner in which these compounds cause greater growth. Perhaps the change in the flora of the intestinal tract due to the antibiotic may be of importance; however, the well-recognized effect of increased intake of food is certainly a primary causative agent in producing greater gains and greater efficiency of feed utilization.

Experiment two

The plan in this experiment was to follow essentially the same line of approach as in the first experiment except that in place of the crude vitamin B_{12} supplement, purified prepa-

rations of the main constituents of this supplement would be substituted. By such a procedure it was hoped that the influence of each of these constituents on growth and efficiency of utilization of the ration could be determined.

The amounts of vitamin B_{12} and streptomycin incorporated in the basal ration were in the same proportion as when the crude B_{12} supplement was used. In this way a new variable was not introduced in the experiment. It was estimated that the supplement supplied 33 μg of vitamin B_{12} activity per kilogram of feed and 5.5 mg of streptomycin per kilogram.

Three rations were used, one containing 7.3 mg of streptomycin sulfate per kilogram of basal ration, this being equivalent to 5.5 mg of streptomycin; the second ration containing 6.6 mg of B_{12} oral grade solids [3] per kilogram, this having a vitamin B_{12} activity of 33 mg; and the third ration containing the above amounts of both B_{12} oral grade solids and streptomycin.

These are known as rations 1, 2 and 3, in the order named. The basal ration is the same as in experiment 1 and as shown in table 1; however, the basal ration was not fed in this experiment without the addition of one of the supplements. The experimental subjects were weanling male albino rats, 12 animals on each ration. They were selected from litter-mate animals, one animal from each litter being placed on one of the three experimental rations, and the feed intake was on a measured ad libitum basis for all groups. Ad libitum feeding was used in this case as it was comparable with the methods employed in other work reported in the literature on these types of rations. Feces and urine were collected quantitatively and the rat bodies were analyzed for nitrogen and energy at the end of the experiment to complete the body balance method for determination of heat production.

In table 5 are given the gain in weight, including gain in empty live weight, gain in fat and nitrogen, days on experiment and efficiency of gains made by the rats on the three

[3] Product of Chas. Pfizer and Co.

rations. All rats were kept on experiment until they had reached a weight near 262 gm; however, the time required to reach this common weight was very different for the different rations. The addition of vitamin B_{12} cut the time in half. When both streptomycin and vitamin B_{12} were added, gain to the same weight was accomplished an average of two days sooner, and efficiency of gains was also improved as compared with gains made when the only supplement was vitamin B_{12}, but the magnitude of these differences is insufficient to be significant by analysis of variance. It should be recognized, however, that if the number of animals used in such a test were sufficiently large, a small difference such as that obtained by the addition of streptomycin could prove to be significant. These findings are essentially in agreement with the recent work of Sauberlich ('52), in which he found that the inclusion of either penicillin or aureomycin in a completely vitamin-supplemented diet had no effect upon the growth of rats.

By merely adding vitamin B_{12} to the basal ration the efficiency of the gains was improved, as is indicated by a reduction in the amount of feed per gram of gain from 4.84 to 2.86 gm. Not only is this difference in efficiency of gains of interest, but the rapidity of gains is also of considerable significance, indicating the importance of vitamin B_{12} in these processes in a monogastric animal, such as a rat, consuming proteins of plant origin.

The different dietary treatments were again without significant influence on the nitrogen gained, as was the case in the first experiment (tables 5 and 6). Some change was observed in fat deposition but nothing comparable to the results when paired feeding was followed. There seemed to be a trend toward a higher fat content of the body when streptomycin was added. In the paired feeding trial the vitamin B_{12} supplement was associated with a much higher fat deposition, but in this case the method of food allotment may have been of greater influence on the nature of the gain than the antibiotic contained in the B_{12} supplement itself.

Complete information on the distribution and utilization of energy is given in table 7. A contrast is noted again in this table in the various values for the rats receiving only

TABLE 5

Food eaten; gains in live weight, nitrogen and fat; and efficiency of gains

RATION	DAYS ON EXP.	FOOD EATEN	INITIAL LIVE WT.	FINAL LIVE WT.
		gm	gm	gm
1 — Streptomycin	82	982 ± 52	48 ± 0.8	253 ± 4.5
2 — Vitamin B_{12}	41	602 ± 17	48 ± 1.1	258 ± 0.4
3 — Streptomycin + vitamin B_{12}	39	573 ± 11	48 ± 1.0	257 ± 0.5

RATION	DAYS ON EXP.	GAIN IN EMPTY BODY WEIGHT	NITROGEN OF BODY GAIN	FAT GAINED	FOOD/ GAIN
		gm	gm	gm	
1 — Streptomycin	82	195 ± 3.9	6.7 ± 0.1	19.35 ± 1.0	4.79 ± 0.3
2 — Vitamin B_{12}	41	200 ± 1.0	6.7 ± 0.1	17.48 ± 1.2	2.87 ± 0.1
3 — Streptomycin + vitamin B_{12}	39	199 ± 1.0	6.6 ± 0.1	18.02 ± 1.1	2.74 ± 0.1

TABLE 6

Distribution of food nitrogen as affected by vitamin B_{12} and streptomycin

DIET AND SUPPLEMENT	1 Streptomycin	2 Vitamin B_{12}	3 Streptomycin + vitamin B_{12}
Nitrogen			
Food, gm	28.6	17.6	16.6
Feces, gm	4.7 ± 0.3	3.0 ± 0.1	2.8 ± 0.1
Digested, gm	23.9	14.6	13.8
% of food N	83.6 ± 0.3	83.0 ± 0.2	83.1 ± 0.2
Urine, gm	16.8	7.7	7.1
% of food N	58.7 ± 1.4	43.8 ± 0.8	42.8 ± 0.8

streptomycin as compared with those for the animals receiving vitamin B_{12} in their ration. In all figures except those for the composition of the body, the slow growth rate of these rats is evident with respect to both protein and fat gained in the form of energy.

There was a very close similarity in the proportion of the food which appeared as heat in the rats on diet 1 in this experiment and the control group in experiment 1. In both cases 73% of the food energy was dissipated as heat. Under these circumstances the streptomycin had no noticeable influence on the metabolism. When the streptomycin was added along with vitamin B_{12}, there was about 1% less of the food energy going into heat than when vitamin B_{12} alone was given.

TABLE 7

Distribution of total energy as affected by vitamin B_{12} and streptomycin

RATION AND SUPPLEMENT	1 Streptomycin	2 Vitamin B_{12}	3 Streptomycin + vitamin B_{12}
Energy value in Calories			
Food	3,913	2,400	2,279
Feces	458 ± 25	308 ± 8	293 ± 7
Digested	3,455 ± 182	2,092 ± 59	1,986 ± 36
Urine	156 ± 19	69 ± 6	64 ± 4
Metabolized	3,299 ± 165	2,023 ± 55	1,922 ± 33
Body gain — total	424 ± 13	408 ± 8	412 ± 12
as protein	244 ± 5	246 ± 3	246 ± 2
as fat	180 ± 10	162 ± 10	166 ± 11
Heat production	2,875 ± 164	1,615 ± 57	1,510 ± 33

As in experiment 1, the addition of vitamin B_{12} had a significant influence on the utilization of energy. The growth rate was doubled, with approximately 6% less of the food energy being lost as heat. Further work is in progress using other antibiotics.

SUMMARY

The effects of a vitamin B_{12} supplement, purified vitamin B_{12}, and streptomycin on the utilization of the food protein and energy of an all-plant protein ration were studied in growth and body balance experiments with growing albino rats as subjects.

In a paired feeding experiment in which one animal of each pair received the basal ration supplemented with a crude vitamin B_{12} mixture, feed consumption was severely limited by the animals on the unsupplemented ration and growth to a common predetermined weight required approximately twice as long as when the supplemented ration was fed ad libitum to a third group of animals. Efficiency of gains was of a much higher order on ad libitum feeding than on paired feeding, and the efficiency of gains was greater in the paired animals receiving the vitamin B_{12} supplement. Body gain of energy was 29% greater in the supplemented pair-fed animals than in the unsupplemented animals, and heat production was 3.5% less.

By feeding the two main constituents of the crude supplement used — vitamin B_{12} and streptomycin — both singly and in combination, but in the same amounts as when the crude supplement was employed, it was found that streptomycin had little effect on growth; vitamin B_{12} greatly improved growth and utilization of feed; and streptomycin and vitamin B_{12} together were slightly better than vitamin B_{12} alone, although no combination was quite as good as the crude supplement itself.

In these studies the nitrogen utilization did not appear to be specifically affected by any of the supplements used.

The more rapid and more efficient growth of the experimental animals receiving vitamin B_{12} is attributed primarily to greatly increased intake of feed as compared to the intake when this supplement was not given.

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EFFECT OF PROTEIN LEVEL AND PENICILLIN ON GROWTH AND INTESTINAL FLORA OF CHICKENS

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FIVE FIGURES

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Considerable information has been compiled concerning the microflora of domestic fowl. King ('05) found the colon bacillus to be the predominating type in the intestinal mucosa of chickens. Rahner ('01) and Gage ('11) showed *E. coli* to be the most numerous species of bacteria in the feces of chickens. Emmel ('30) concluded from his experiments that *E. coli* and *E. communior* were the most important species, making up 34 and 31% of the total flora, respectively. Harrison and Hansen ('50) showed that 50% of the total cultivable flora of cecal feces of turkeys was made up of anaerobic lactobacilli, Gram-positive, non-sporeforming rods other than lactobacilli, and that anaerobic streptococci were next in predominance. Micrococci were found to be variable. Coliforms and enterococci existed in relatively small numbers, usually comprising less than 1%. It was also evident that, in the cecal flora, anaerobic bacteria were in the majority and that almost the entire flora was composed of Gram-positive organisms.

Work by Porter and Rettger ('40) on the influence of diet showed that the so-called "normal" bacterial flora can be altered throughout the digestive tract by certain foods or starvation, but that it is fairly stable, irre-

spective of diet. Once the flora has been changed, it can be transformed back to "normal" by administration of appropriate diets. Johansson et al. ('48) observed a change in the intestinal flora of hens associated with a change of diet. The organisms were identified mainly as the bacterial types, coliform, enterococci, lactobacilli, and so forth. Their data suggest that anaerobic lactobacilli exist in the intestines of chickens in considerable numbers. Moore et al. ('46) found that orally administered streptothricin, streptomycin, sulfasuxidine or a combination of streptomycin and sulfasuxidine failed to sterilize the intestinal tract of chicks, but produced a reduction in the coliform bacteria of the cecal contents.

The purpose of this paper is to report on the alterations in growth and in the bacterial flora, aerobic and anaerobic, in the ceca of chickens, as influenced by diets containing various levels of protein, with and without penicillin.

EXPERIMENTAL PROCEDURE A

Eight groups, each comprised of 76 Barred Plymouth Rock male chicks, were maintained on litter and fed diets containing 17, 20, 23 and 26% protein. The 17% protein diet contained 51% ground yellow corn, 30% wheat shorts, 5% fish meal, 5% meat meal, 5% dried buttermilk, 0.75% ground limestone, 1.25% steamed bone meal, 0.25% iodized salt, 0.5% fish oil (400 D–3,000 A/gm) and 1.25% dehydrated alfalfa meal. In addition, 9 gm manganese sulfate, 200 mg riboflavin, 1 gm niacin, 0.1 mg vitamin B_{12} and 22.7 gm Megasul [1] were added to each 100 lb. of diet. The 20, 23 and 26% protein diets contained 10, 20 and 30% of soybean oil meal, respectively, replacing equivalent amounts of wheat shorts. Each diet was fed in the absence of the antibiotic and in the presence of 10 p.p.m. of penicillin G potassium. The birds were weighed individually at 10 weeks of age and feed consumption was recorded.

[1] A product of Lederle Laboratories, Pearl River, N. Y., designated as containing 25% nitrophenide. The material is used as a coccidiosis preventive.

At the termination of the experiment 24 average birds were sacrificed from each group. They were aseptically opened and the ceca ligatured off. The outer surface was washed with mercuric chloride solution and removed to sterile petri dishes. The cecal contents were expressed by the use of a sterile spatula and forceps into a second series of sterile plates. The samples from 12 birds were pooled in each case, thus giving two samples from each group, amounting to a total of 16 separate pooled samples.

The pH of the various pooled cecal samples was recorded, using a Beckman pH meter and employing contact electrodes applied to the thoroughly mixed sample. For bacteriological examination 10 gm were taken from each pooled sample and added to a 90-ml sterile distilled H_2O dilution blank containing glass beads. The sample was thoroughly shaken on an automatic shaking machine for 5 minutes. From this initial dilution, dilutions of 10^{-3}, 10^{-4}, 10^{-6}, 10^{-8}, and 10^{-9} were made and duplicate 1-ml samples of each transferred to the following media: (a) Levine Eosin methylene blue agar [2] plates used for the isolation of the members of the coliform group; (b) "S.F." broth of Hajna and Perry ('43) in tubes for the dilution counts of enterococci; employing M.P.N. method; (c) tomato juice agar [3] plates overlaid with 2% agar for isolation of lactobacilli; (d) tryptone glucose extract agar with 2% yeast extract [4] added for cultivation of aerobes. T.G.Y.E. agar with B.C.P. and 2% skim milk for isolation of the aciduric and proteolytic types of organisms; (e) Reed's modification of Brewer's thioglycollate medium (Reed and Orr, '43) for cultivation of anaerobes; employing M.P.N. method and 2% agar added to each tube to form a plug.

The above media were incubated at 37°C. for 48 hours, with the exception of "S.F." medium, which was incubated at 41°C. for 72 hours. Representative colonies were picked off and maintained on T.G.Y.E. agar and tomato juice agar

[2] Difco.
[3] See footnote 2.
[4] See footnote 2.

slants and traced out for confirmation of identification as to genera.

EXPERIMENTAL PROCEDURE B

Respiration studies of biological activity on various diets with and without penicillin

In the theory of microbiological respiration of Kluyver and Donker (as reported by Porter, '47) it is stated that "respiration, the process by which cells derive the energy necessary for growth from their substrate, can take place aerobically and anaerobically." In the present experiment only aerobic respiration has been studied.

In the following experiments, using the Warburg respirometer, small samples of diets were required. The gas volume of the Warburg flasks was known, and since the addition of the diet changed the volume, the specific gravity of the samples was necessary so that correct compensation could be made when determining the flask constant. The pycnometer method for determining specific gravity described by Lyon and Buckman ('47) was employed. Flask constants were determined according to the "Manometric studies" of Umbriet et al. ('49).

Fluctuations of temperature were eliminated by using a thermostatically controlled water bath, and thermobarometers compensated for changes in barometric pressure. To measure O_2 uptake, KOH was required in the centre well of the flask to absorb CO_2. Small strips of filter paper were inserted in the well with the KOH to increase the absorption surface.

Duplicate 1-gm samples of the various diets were introduced into the Warburg vessels. One milliliter of sterile distilled H_2O was added to the controls, while to the duplicate in each case was added 1 ml of a suspension of resting cells of bacteria under study. In each case the bacteria were isolated from the cecal contents of the chickens on experiment. The suspensions of cells were made from 24-hour cultures of these bacteria, which were repeatedly washed and centrifuged in sterile physiological saline solution until all nutrients were

eliminated. Serial dilutions were made and plated out in order to obtain the required number of cells per milliliter of inoculant. A suspension of approximately 5×10^5 cells was employed throughout.

After addition of KOH, filter paper, diet, sterile H_2O and inoculant the flasks were randomized, attached to their respective manometers and placed in one of two banks in the thermostatically controlled water bath at 37°C. A period of adaptation was required and adaptation was established within 30 minutes. The manometer cocks were closed and readings taken periodically over a three-hour period.

RESULTS

The weight and feed efficiency data are presented in figure 1. The weight data were examined statistically according to the method of analysis of variance. The effects of protein level and penicillin were found to be highly significant ($P<0.01$). While interactions between the level of protein and penicillin were not significant, an examination of the individual mean weights and feed efficiencies suggests that there was a greater response from penicillin with the 26% protein diet than at lower levels. It would appear that, in the absence of the antibiotic, the highest level of protein tended to depress growth and feed efficiency as compared to the diet containing 23% protein. The depressive effect of the high level of protein appeared to be considerably relieved by the presence of penicillin in the diet.

The average pH values of duplicate samples of the cecal contents of chicks fed diets containing 17, 20, 23 and 26% protein were 6.32, 6.30, 6.28 and 6.23, respectively. When penicillin was included in the above diets, the respective pH values were as follows: 5.90, 5.70, 5.61 and 5.62. It will be noted that increasing the level of protein in the diets tended to reduce the pH of the cecal contents. This was true particularly in the presence of the antibiotic when the protein level was increased from 17 to 23%. Penicillin effected a considerable lowering of pH with all diets.

The data concerning viable bacterial counts are presented in figure 2. These results indicate that, in general, in the absence of penicillin the protein level failed to alter the

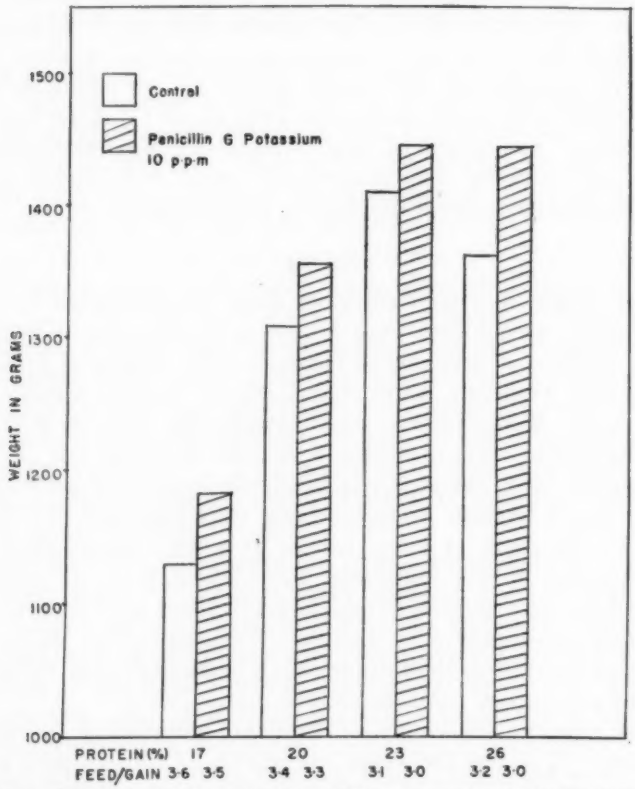

Fig. 1 Effect of protein level and penicillin on growth and feed efficiency of chickens.

counts materially. However, anaerobes and microaerophiles tended to increase as the protein was raised from 17 to 23%. Also, there is a suggestion that the proteolytic count was increased somewhat at the 26% level of protein. With diets containing up to 23% protein, penicillin caused an increase

in all counts except that for enterococci. In general, highest counts were encountered in the birds fed the 23% protein diet in the presence of penicillin.

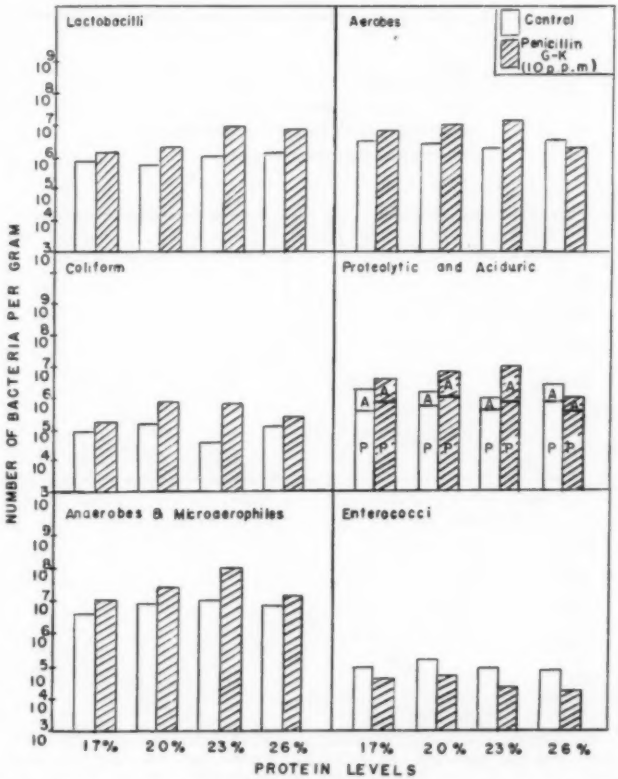

Fig. 2 Effect of protein level and penicillin on cecal bacterial counts.

Enterococci counts were reduced by penicillin at all levels of protein. The decrease in numbers of these organisms did not appear to be influenced by the protein content of the diet. The increase in numbers of coliform organisms resulting from the addition of penicillin to these diets was due to the presence of an atypical strain of *E. coli*. This strain lacked

the characteristic green metallic sheen, fermented lactose slowly, did not attack dulcitol and comprised 92% of the total coliform count under these conditions.

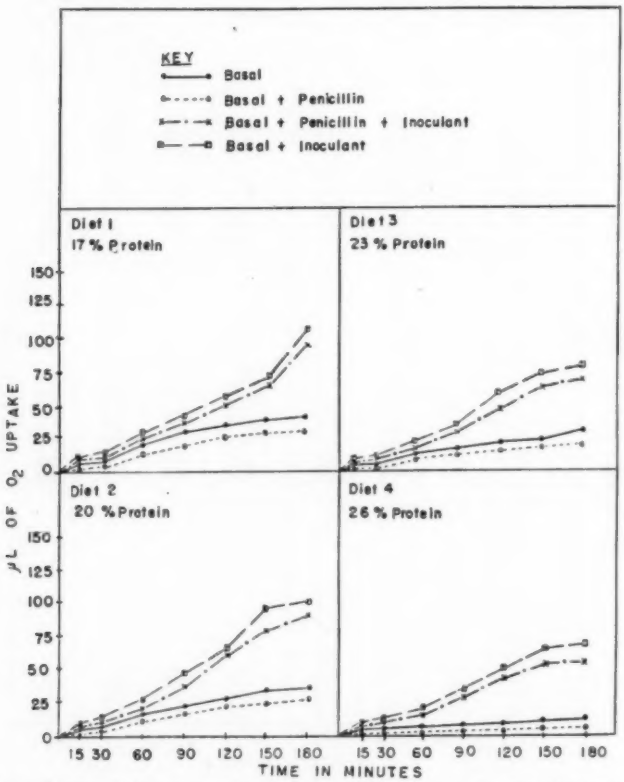

Fig. 3 Manometric measurement of utilization of various protein levels with and without penicillin by *E. coli*.

The relative size of the ceca of groups fed the various diets was noted as the birds were opened to obtain cecal samples. The most striking observation was the great increase in cecal size caused by penicillin. This effect was readily apparent with all levels of protein.

Bacteriological analyses of the various diets which were used for both the feeding experiment and respiration studies revealed the absence of coliforms, lactobacilli and enterococci.

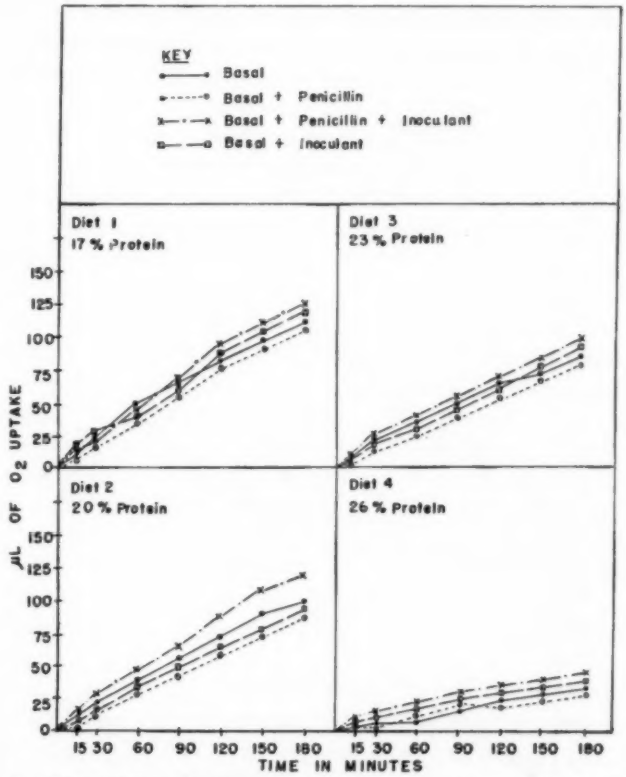

Fig. 4 Manometric measurement of utilization of various protein levels with and without penicillin by a lactobacillus organism.

However, aciduric types were present in the 10^{-3} dilution and proteolytic types in the 10^{-4} dilution. Molds and spore-formers were quite numerous in all diets.

The results of respiration studies using *E. coli* as the inoculant are presented in figure 3. The presence of the

inoculant at all levels of protein increased respiration considerably. Penicillin reduced O_2 uptake in both the presence and absence of the inoculant to about the same extent. This would suggest that the *in vitro* respiration of *E. coli* was not materially influenced by the antibiotic.

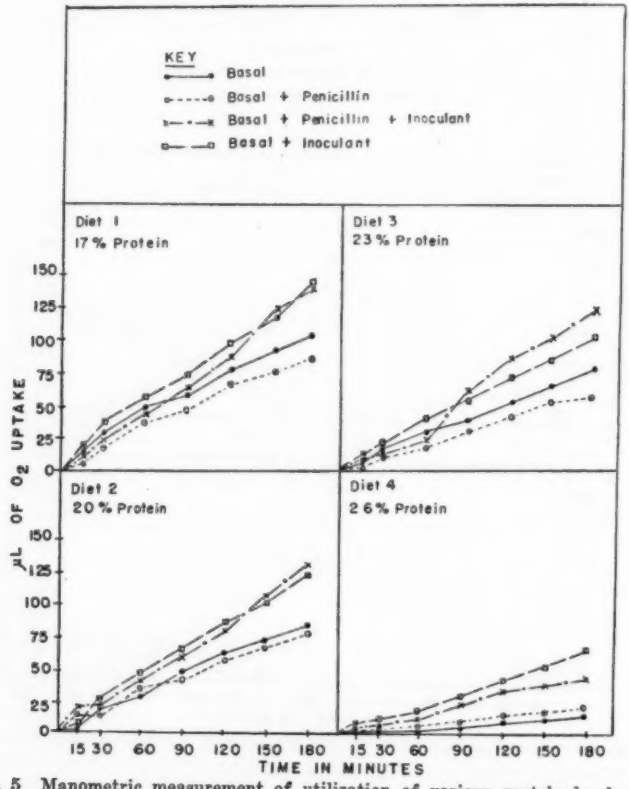

Fig. 5 Manometric measurement of utilization of various protein levels with and without penicillin by an aciduric organism.

In studies using atypical *E. coli* and a proteolytic type of organism, the results were similar to those obtained with *E. coli* as the inoculant. These data, therefore, will not be presented in detail.

The results using lactobacillus as the inoculant are presented in figure 4. As in the case of *E. coli* and atypical *E. coli,* penicillin reduced the O_2 uptake of the control diets. However, in contrast to the results with these organisms, penicillin increased the respiration when added to all diets inoculated with lactobacillus. This suggests that penicillin had a favorable effect on the diet as a medium for this organism. It will be noted that the O_2 uptake for the control diets and for these diets with penicillin are not wholly in agreement in respiration tests using all organisms. This discrepancy may be attributable to the fact that the organisms were studied on different days and different samples of the diets were used.

The respiration data using an aciduric type organism as the inoculant are shown in figure 5. In considering the diets containing up to 23% protein, the results indicate a tendency on the part of penicillin, when added to diets containing the inoculant, to improve O_2 uptake. There was a period of adaptation in all cases. However, the duration of this period was short with the 23% protein diet and the antibiotic effect was marked. These data indicate that the aciduric type of organism was influenced in a favorable manner, particularly with the 23% protein diet. These results are interesting in view of the fact that penicillin had its greatest effect on this organism with the same diet in the cecal flora studies (fig. 2).

DISCUSSION

The weight and feed efficiency data indicate that optimum results were achieved with the 23% protein diet and that penicillin did not alter the protein requirement under these conditions. On the other hand, the tendency of penicillin to relieve the depressive effect of the highest level of protein suggests that the antibiotic enhanced protein utilization and increased the tolerance to this nutrient.

In general, the reductions in pH of the cecal contents caused by protein and penicillin appear closely associated with the increase in numbers of lactobacilli and aciduric bacteria. It

is of particular interest that the difference in pH of the cecal contents of birds fed diets with and without penicillin increased with elevation of the protein up to a level of 23%. These changes are also apparent in the counts of lactobacilli and of aciduric types of organisms.

A possible mechanism for the pH and bacterial changes caused by penicillin may be postulated as follows: The increasing growth of aciduric rather than proteolytic types of organisms appeared to result in a more favorable medium. It was found in these studies that less than 10% of the aerobes had the ability to break down gelatin. It may be noted that the anaerobes and microaerophiles increased with increasing protein up to a level of 23% and with the addition of penicillin. A marked proteolytic activity was demonstrated by these organisms. It may be that, after autolysis and initial degradation of protein, the anaerobes become active and create a slightly acid medium. As a result of the action of the anaerobes, suitable protein by-products and a more favorable pH would be established for the growth of lactobacilli and the aciduric type of organisms. The metabolism of these organisms would tend to cause a further decrease in pH and consequent inhibition of the growth of the proteolytic or putrefactive organisms. This might well account for increased utilization of protein and a raising of the tolerance to this nutrient.

It has been reported by Moore et al. ('46) that the coliform count of the cecal contents was reduced by antibiotics. In the present study coliform counts increased in the presence of penicillin. However, it was shown that the amount of *E. coli* was reduced and that the increase in coliform counts was due to the presence of an atypical strain of *E. coli*. This strain would appear to be a resistant mutant of *E. coli*. Enterococci counts were reduced by penicillin, suggesting that this antibiotic restricted such organisms as Gram-positive staphylococci and streptococci. The reduction in these counts was similar with all levels of protein and did not appear to be related to growth. These data, together with the bacterial

counts of desirable types, constitute evidence that the increase in the growth of chickens caused by penicillin is not entirely due to inhibition of pathogenic organisms.

With diets containing levels of protein up to 23% a relatively close correlation may be noted between increased growth of the birds and elevation in the numbers of certain microorganisms. This relationship did not appear to hold for the 26% protein diet. These data suggest that while increased numbers of desirable organisms may be a factor in causing the growth response to penicillin, such an explanation is not adequate for all cases. On the other hand, it may be that penicillin caused a greater increase in the size of the ceca with the 26% protein diet than with lower levels, and that total counts would have given a truer picture.

As to importance in the mechanism of growth response to the antibiotic, the increase in microorganisms may be only incidental to the increase in cecal size. The considerable increase in the size of the ceca caused by penicillin might well afford sufficient additional absorptive capacity to enhance the utilization of protein and all other nutrients. On the other hand, the reduction in pH may be an important consideration in explaining growth response to the antibiotic. The lowered pH could conceivably increase the activity of the villi of the small intestine or result in more complete enzymatic digestion.

In respiration studies using specific organisms isolated from the cecal contents, it was found that only in the case of lactobacillus and the aciduric type of organisms did penicillin cause an increase in O_2 uptake. It may be noted that the O_2 uptake response to penicillin was greatest in the case of the 23% protein diet with the aciduric type of organism as the inoculant. This is of interest in view of the similar results on cecal bacterial counts, and suggests that the suitability of this diet, *in vitro* as well as *in vivo,* for the growth of aciduric types is enhanced by penicillin.

SUMMARY

Penicillin caused an increase in weight and improvement in feed efficiency of chickens fed diets containing 17, 20, 23 and 26% protein. While penicillin did not appear to change the protein requirement, the evidence suggests that the antibiotic enhanced protein utilization.

A considerable reduction in the pH of the cecal contents of chickens resulted from the addition of penicillin to diets containing 17, 20, 23 and 26% protein. The extent of the reduction increased with increasing protein up to the 23% level. The alterations in pH appeared to be associated with the increase in numbers of lactobacillus, aciduric, anaerobic and microaerphilic, aerobic and coliform types of organisms.

The increase in coliforms was due to the presence of an atypical strain of *E. coli*. Enterococci counts were reduced by the addition of penicillin, the reduction being similar at all levels of protein. Penicillin effected a marked increase in the size of the ceca with all levels of protein.

In *in vitro* respiration studies using the various diets as substrate and the specific organisms as inoculant, penicillin enhanced the O_2 uptake of lactobacillus and the aciduric type of organisms.

With diets containing levels of protein up to 23% a relatively close correlation was noted between increased growth of the birds and elevation in the numbers of certain microorganisms. It is suggested that the increase in numbers of these microorganisms or the resultant increase in the size of the ceca may be factors in explaining the mechanism of growth response to the antibiotic. On the other hand, the decrease in pH may cause increased activity of the intestinal villi or more complete digestion, and thus play a role in the growth response mechanism.

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RELATION OF SERUM CHOLINESTERASE TO NUTRITIONAL STATUS OF ADOLESCENTS

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McCance et al. ('48) reported that the mean serum cholinesterase levels in 82 German workers subsisting on civilian rations in 1946, and in 57 men with nutritional edema, were approximately half the normal values. When the malnourished men were given enough food to produce a considerable increase in body weight, the serum cholinesterase approached normal. A subsequent restriction of food and loss of body weight reduced the cholinesterase levels again. These findings led the authors to conclude that the serum cholinesterase level was a delicate index of the state of nutrition of any group of men in Germany at that time. A detailed report of this study has recently appeared (Hutchinson et al., '51). Waterlow ('50) has reported that the serum cholinesterase in malnourished infants was very much lower than that for healthy infants.

Since current methods of nutritional assessment provide no generally accepted method of evaluating the over-all condition of an individual, it appeared desirable to determine

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the relation of serum cholinesterase to some of the accepted parameters of nutritional status. The need for such an evaluation was further prompted by the simplicity of the technique and the claims made by McCance and his group for the specificity of serum cholinesterase levels.

The present work compares the serum cholinesterase level with other measures of nutritional status made on the same children.

EXPERIMENTAL

One hundred and nineteen children in two junior high schools in Washington, D. C., were selected by the school nurses and principals as being malnourished. Two schools in an economically impoverished area were chosen, since it was anticipated that malnutrition would be more prominent in them. One of the schools was for Negroes, the other for whites. If the study had showed any suggestive correlations between cholinesterase blood levels and nutritional conditions, an additional group of well-nourished students would have been studied.

The distribution of the children according to age, sex and race is given in table 1.

Each child was examined by a physician who had specialized in clinical human nutrition. Special emphasis was put on lesions of the skin, mouth and eyes. Thirty-nine localized regions were examined and graded from zero, showing the absence of abnormality, through three, which indicated a severe disturbance (Sandstead and Anderson, '47). Anthropometric measurements were restricted to the determination of height and weight. A sample of blood was obtained by venipuncture. Hemoglobin was determined by the method of Turner ('46), carotene by the method of Bessey et al. ('46), and serum cholinesterase by the Michel method ('49) as modified by Vorhaus and Kark ('49). The blood for the cholinesterase determinations was kept in a refrigerator until the analyses were made. All of the cholinesterase analyses were completed within a week after the samples were collected.

The study was done in late winter. Each day the children reported for the examination at regular intervals after arriving at school. The work was usually completed before the noon meal. The blood samples were divided into two portions; one was placed in a tube containing mixed oxalates (ammonium and potassium), the other in a clean, dry tube. Hemoglobin was determined on the uncoagulated whole blood, while serum was used for the other determinations.

TABLE 1

Age, race and sex of the children in the study (Washington, D. C., January, 1950)

SEX	TOTAL	AGE GROUP						
		11	12	13	14	15	16	17
				Both races				
Grand total	119	1	17	26	39	26	9	1
				Whites				
Total	34	0	4	9	6	12	3	0
Boys	14	0	3	1	2	6	2	0
Girls	20	0	1	8	4	6	1	0
				Negroes				
Total	85	1	13	17	33	14	6	1
Boys	28	0	1	3	12	8	3	1
Girls	57	1	12	14	21	6	3	0

In order to determine statistically if there were any correlation between cholinesterase values and other measures of nutritional status, the product moment method for ungraded data was used for per cent underweight as based on the tables of Baldwin and Wood ('23), the number of physical findings suggestive of malnutrition (excluding findings on general appearance, teeth and weight), carotene and hemoglobin values. The correlation between cholinesterase and general appearance, rated as fair and good, was evaluated by comparing the mean values for the different observations on the children in the two groups.

RESULTS

According to the hemoglobin and serum carotene standards proposed by Bessey and Lowry ('47), the children in the present study (table 2) were in "fair" nutritional condition.

The biochemical findings for the children separated by the physician into "fair" and "good" nutritional groups were the same except for the degree of underweight and the serum carotene levels (table 3). The carotene values were actually a little higher in the group rated "fair" than in

TABLE 2

Physical and biochemical results

	VALUES		NO. CHILDREN STUDIED [1]
	Mean	Std. Dev.	
Cholinesterase (pH units/hr.)	0.96	± 0.24	103
Underweight (%)	11.90	± 5.57	103
Physical findings (no.)[2]	3.29	± 2.63	112
Carotene (μg/100 ml)	95.95	± 33.80	93
Hemoglobin (gm/100 ml)	12.60	± 1.45	112

[1] The number of children in each group was limited as follows: (a) the underweight group was restricted to children who were 5% or more below their standard weight as given in the Baldwin and Wood ('23) tables; and (b) insufficient blood was secured in some cases for all the analyses.

[2] The number of physical findings refer to abnormalities of skin, eyes, lips, tongue, and so forth, examined for signs indicative of malnutrition; for details, see Sandstead and Anderson ('47).

the "good" group. The cholinesterase values were the same for the "good" and "fair" groups. There was only one child in the "poor" category; his data were omitted from the comparison.

The only statistically significant correlations were the relation of serum cholinesterase to the degree of underweight and level of serum carotene (table 4). The lower the body weight in relation to the standard weight (from Baldwin and Wood tables), the higher was the serum cholinesterase level. This relationship was seen, however, only when the group

was limited to the children who were 5% or more below their standard weight. McCance et al. ('48) and Hutchinson et al. ('51) reported the opposite relation between serum cholinesterase and underweight. On the other hand, the higher the serum carotene level, the higher was the serum cholinesterase.

TABLE 3

Relation between serum cholinesterase levels and physical appraisal of the subjects[1]

PHYSICAL APPRAISAL	SUBJECTS		SERUM CHOLINESTERASE		UNDERWEIGHT		SERUM CAROTENE		HEMOGLOBIN	
	Race	No.	Units	S.E.	%	S.E.	μg	S.E.	gm	S.E.
Good	W	16	0.98	0.055	11.1	2.2	89.5	9.7	13.5	0.21
	N	52	0.92	0.019	8.6	1.1	94.1	3.9	12.6	0.22
Fair	W	11	1.04	0.078	15.3	1.8	100.8	8.9	13.7	0.29
	N	13	0.92	0.055	15.2	2.3	107.7	11.0	12.4	0.48

[1] Only those children for whom a complete set of data existed were used in this comparison.

TABLE 4

Relation between serum cholinesterase levels and measures of nutritional status

	CORRELATION COEFFICIENTS
Cholinesterase × underweight	0.21[1]
Cholinesterase × physical findings	0.02
Cholinesterase × serum carotene	0.25[2]
Cholinesterase × hemoglobin	0.15

[1] Significant at the 2 to 5% level.
[2] Significant at the 1 to 2% level.

There was no significant difference in the cholinesterase values for the Negro and white children. The mean value for the Negro children was 0.94 pH units per hour, while that for the white children was 1.02. The mean for the entire group was 0.96 pH units per hour (table 2), which corresponds to the mean of 0.94 for 68 normal individuals reported by Vorhaus and Kark ('48) using the same analytical procedure.

DISCUSSION

The relation between serum cholinesterase and nutritional status as measured by the parameters used in this study is such that the serum enzyme level contributes nothing toward the nutritional appraisal of people in a condition similar to that seen among Americans. Although there was a statistically significant correlation between the degree of underweight and the serum cholinesterase level (table 4), there are a number of factors that nullify the use of this enzyme in nutritional evaluations. In the first place, the correlation, as determined on the basis of the underweight children, is positive. As the degree of underweight increases, so does the serum cholinesterase level. This is opposite to the relation reported by McCance et al. ('48) and Hutchinson et al. ('51). Secondly, the correlation coefficient was statistically significant only when the children who were of normal weight or above were excluded from the calculation. If the entire group were evaluated on the basis of cholinesterase levels, as one would a population sample, then there would be no correlation between the enzyme level and degree of underweight ($r = 0.09$). Finally, even if calculation of the correlation coefficient were limited only to underweight children, the serum cholinesterase would offer little help in evaluating nutritional status. In the present study 67% of the white children and 63% of the Negro children were 10% or more underweight according to the Baldwin-Wood tables. Some of the children were as much as 29% underweight. In spite of this rather large spread in the degree of underweight, there is a relatively narrow range for the cholinesterase levels encompassed within the regression line. The regression line extends from a cholinesterase value of 0.87 pH units per hour for the children who were 0% underweight to 1.10 pH units per hour for the children who were 30% underweight. The spread of these values is only a small segment of the levels found in these children (range = 0.41 to 1.57). Any cholinesterase value may be associated with

a rather wide range of leanness as determined by measurements of height and weight.

There was a positive correlation between the serum cholinesterase and carotene levels. As the serum carotene level increased, so did the cholinesterase values. This is what one would have expected on the basis of the work of McCance et al. ('48) and Hutchinson et al. ('51). Unfortunately, the regression line for these two variables is also very nearly parallel to one axis. The regression line goes from a cholinesterase value of 0.84 pH units per hour for the child with 20 μg carotene per 100 ml of serum to 1.07 pH units per hour for the children with 180 μg carotene per 100 ml of serum. It would be extremely difficult to deduce very much about the vitamin A status of an individual from the cholinesterase level of his serum. Part of that difficulty can be attributed to the confusion surrounding the interpretation of serum carotene levels (Caster, '52; Medical Research Council, '49).

Table 3 permits an evaluation of the factors that might have entered into the over-all nutritional ratings assigned the subjects by the physicians. Both groups had the same hemoglobin levels and essentially the same serum carotene and cholinesterase levels. The most prominent difference between the two groups was that the "fair" group showed a higher percentage of underweight than the "good" group.

There are indications that the level of hemoglobin is more intimately related to the body's supply of protein than is the plasma protein level (Keys et al., '50). The poor success attained when serum protein determinations have been used in the assessment of nutritional status led to the use of hemoglobin levels in the present study. The only significant finding with respect to the hemoglobin values was a difference in the levels of white children (13.5 gm per 100 ml) and Negro children (12.6 gm). The difference between the two groups is statistically significant ($t = 4.05$). Differences of that magnitude in the hemoglobin levels between Negroes and white have been reported by a number of investigators

(Nutrition Branch and Program Analysis Branch, '49). The absence of any relation between hemoglobin levels and serum cholinesterase was confirmed by the data of Sawitsky et al. ('48). In spite of a wide range of cholinesterase levels, the hemoglobin values for their 15 subjects showed no relation to the enzyme levels.

Both reports from the Cambridge workers (McCance et al., '48; Hutchinson et al., '51) emphasized the fact that the "pseudo" cholinesterase in the serum changed with nutritional status to a greater extent than did the "true" cholinesterase. The method used in the present study (Michel, '49) measures primarily the "pseudo" cholinesterase, since the "true" cholinesterase is relatively inactive at the high concentration of acetyl choline used in the determination (Mendel and Rudney, '43).

The difference between our findings with children and those reported by McCance et al. ('48) and Hutchinson et al. ('51) for adults cannot be attributed to differences in age. McCance and his group ('49) showed that the adult cholinesterase level in the serum was attained by the 21st day of life. Furthermore, a report by Milhorat ('38) indicated that serum cholinesterase is not associated with age, sex, body weight or muscle mass.

A decreased serum cholinesterase level is associated with liver disease. It is effective in the differential diagnosis of hepatocellular disease and uncomplicated obstructive jaundice (Vorhaus and Kark, '49). It is difficult to determine whether the subjects studied by Hutchinson et al. ('51) were the same as those examined by Sherlock and Walshe ('51) with respect to liver functions. They were all from the same large experimental group. On the basis of rather extensive tests, Sherlock and Walshe were unable to find any signs of liver disturbances among the malnourished Germans they studied. Under these circumstances, it is difficult to attribute the low serum cholinesterase levels secured by McCance et al. ('48) and Hutchinson et al. ('51) to liver abnormalities.

There may be some argument as to whether the underweight children studied in Washington are comparable to the persons seen by McCance et al. ('48) and Hutchinson et al. ('51) in Wuppertal, Germany, in 1946. The children in the present study are, however, fairly representative of the malnutrition which is presently encountered in the United States. Recent surveys have revealed only a small percentage of the people in this country to be severely malnourished. The characterization of their condition has always been a relatively simple task. The pressing need in nutrition is to find unequivocal methods of assessing the condition of the less severely malnourished.

One of the apparent drawbacks to the use of cholinesterase levels as a measure of any physiological function is the great individual variation. Normal individuals may show as much as a 4-fold range in plasma cholinesterase levels (Sawitsky et al., '48). The great variation in normal serum cholinesterase values may help explain the low correlations between that function (cholinesterase levels) and other measures of nutritional status.

SUMMARY

In 119 children no useful relation was found between serum cholinesterase levels and hemoglobin, serum carotene, degree of underweight, physical signs indicative of malnutrition, or general appearance from a nutritional standpoint. The lower the body weight in relation to the standard weight (from height-weight tables), the higher was the serum cholinesterase level, which is the reverse of the relationship previously reported. On the other hand, the higher the serum carotene level, the higher was the serum cholinesterase. Since the range of cholinesterase levels encompassed by the regression line was very small in both cases, and the range of cholinesterase levels in normal children was as great as that in malnourished children, the application of this enzyme level to nutrition studies is of dubious value among populations at nutritional levels similar to those investigated in this study.

ACKNOWLEDGMENTS

This study was made after consultation with Dr. Daniel L. Seckinger, Director of Public Health, and Dr. Richard L. De Saussure, Director of School Medical Inspection Service, District of Columbia Health Department. We wish to thank the District of Columbia Board of Education for approving the project. Mr. Hugh J. Smith and Mr. Ernest Jenkins, principals of the schools where the children were examined, aided greatly in making the study possible. Dr. Fred Morse and Dr. Stanley Tauber assisted with the physical examinations and Miss Dorothy Kremen aided in the blood analyses. Mrs. Georgia Druzzina and Mr. Marvin A. Schneiderman helped with the statistical evaluation of the data.

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BIOTIN DEFICIENCY IN SUCKLING PIGS [1, 2, 3]

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THREE FIGURES

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McRoberts and Hogan ('44) have reported that there is no evidence that the pig requires a dietary source of biotin. However, while a dietary requirement for biotin has not been demonstrated, it has been shown that the pig does utilize the vitamin. Cunha, Lindley and Ensminger ('46) produced a deficiency syndrome in weanling pigs on a purified diet in which desiccated egg white was the source of protein. The alopecia, dermatitis, cracked feet, and spasticity of the hind legs reported as deficiency symptoms were effectively prevented by the daily intramuscular injection of 100 μg of biotin.

Lindley and Cunha ('46) found no beneficial effects when biotin was added to a ration containing thiamine, riboflavin, niacin, pyridoxine, pantothenic acid, and choline. When sulfaguanidine was added to the ration, deficiency symptoms simi-

[1] Published with the approval of the Director of the Idaho Agricultural Experiment Station as research paper 339.

[2] We are indebted to Dr. D. F. Green, Merck and Co., Rahway, N. J., for supplies of thiamine, riboflavin, pyridoxine, nicotinic acid, biotin, inositol, choline, *p*-aminobenzoic acid, calcium pantothenate and alpha-tocopherol; to Dr. E. L. R. Stokstad, Lederle Laboratories, Inc., Pearl River, N. Y., for pteroylglutamic acid; and to Dr. J. Waddell, E. I. du Pont de Nemours Co., New Brunswick, N. J., for crystalline vitamin D_3.

[3] The authors wish to acknowledge the assistance of Mr. W. V. Hartwell and Mrs. June Anderson in these studies.

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lar to those reported by Cunha, Lindley and Ensminger ('46) were observed. Colby and associates ('48) showed that the inclusion of biotin in the ration of a pantothenic acid-deficient pig was effective in prolonging the animal's life, but caused the pantothenic acid deficiency symptoms to appear in half the time.

TABLE 1

Composition of ration

COMPONENTS	AMOUNT	VITAMINS ADDED [2]	LIQUID MILK
	%		*mg/kg*
Casein	30.0	Thiamine	0.65
(Labco)		Riboflavin	0.65
Cerelose	37.4	Nicotinic acid	2.50
		Inositol	26.00
Lard [1]	26.6	Choline	260.00
		p-Aminobenzoic acid	2.60
Salts [2]	6.0	Pteroylglutamic acid	0.052
		Pyridoxine	0.65
		Calcium pantothenate	1.30
		Alpha-tocopherol	1.00
		2-Methyl-1,4-naphthoquinone	0.28
		Vitamin A	2,000 I.U./kg
		Vitamin D	200 I.U./kg

[1] The lard was homogenized with the solution of casein, cerelose and salts to produce an emulsion containing 13% solids.

[2] Modified salt mixture of Phillips and Hart ('35).

[3] Control animals also received 0.01 mg biotin per kilogram liquid diet.

Since the literature relative to the growing pig's need for biotin in its ration is incomplete the experiments herein reported were undertaken to add to available information.

EXPERIMENTAL PROCEDURE

Three Chester White (trial 1) and 4 Duroc (trial 2) suckling pigs which were permitted to receive colostrum for 48 hours were used as experimental animals. The pigs in both trials were litter mates and were kept in individual metal metabolism pens having screen floors designed to separate the urine and feces. The experimental ration (table 1) was

TABLE 2

Urinary excretion of biotin [1]

AGE IN DAYS	TRIAL I					TRIAL II					
	Control		Deficient			Control		Deficient			
			Pig 9		Pig 10			Pig 23		Pig 24	Pig 25
	Intake	Excretion	Intake	Excretion	Excretion	Intake	Excretion	Intake	Excretion	Excretion	Excretion
	μg/day	*μg/day*	*μg/day*	*μg/day*	*μg/day*	*μg/day*	*μg/day*	*μg/day*	*μg/day*	*μg/day*	*μg/day*
2	4.0	0.3	0	0.4	0.2	3.0	1.2	0	1.0		1.3
9	6.0	0.3	0	1.0	0.2	6.0	1.4	0	1.6	0.6	0.4
15						12.0	1.5	0	2.2	1.6	1.8
21	11.0		0	0.6	1.6	16.0	2.3	0	2.3	1.7	1.6
27						24.0	3.7	0	3.2	2.9	2.7
28						24.0	4.2	0	1.6	1.4	1.4
29 [2]	18.0	1.9	0	2.0	2.7						
35						42.0	8.4	0	5.3	2.5	4.9
39	24.0	3.9	0	2.4	3.1						
42 [3]						51.0	10.0	0	2.4	1.1	1.5
48 [4]	30.0	6.8	10	3.4	3.3	60.0	12.2	0	1.1	0.5	0.6
56	42.0	15.0	10	2.9	3.5	78.0	9.0	0	0.5	0.3	0.3

[1] Microbiological assay using *Lactobacillus arabinosus*, modification of the procedure of Shull, Hutchings and Peterson ('42).
[2] Began feeding pigs 9 and 10 sulfathalidine at the rate of 0.5 gm per feeding (3 gm per day).
[3] Began feeding pigs 23, 24, and 25 sulfathalidine at the rate of 1 gm per feeding (6 gm per day).
[4] Ceased feeding sulfathalidine to pigs 9 and 10.

a "synthetic milk" lacking biotin. The control ration contained 0.01 mg of biotin per kilogram of liquid diet. The "synthetic milk" was similar to that reported by Wiese et al. ('47) for calf nutrition studies, with modifications for suckling pigs as described by Lehrer et al. ('49). All feeding was done with nursing bottles to prevent contamination of urine and to permit accurate feed intake records to be made (table 3).

Urine samples were collected at periodic intervals. The biotin excretions (table 2) were determined by a modification of the method of Shull, Hutchings and Peterson ('42), using *Lactobacillus arabinosus.*

RESULTS AND DISCUSSION

General observations

Suckling pigs started on a biotin-deficient purified diet when the pigs were 48 hours old developed certain deficiency symptoms which were attributed to a lack of this vitamin (fig. 1). The results of this experiment are not in accord with those of McRoberts and Hogan ('44) and Lindley and Cunha ('46), who used 56-day-old pigs that had already developed an intestinal flora which apparently synthesizes biotin in adequate amounts for normal body function. The symptoms associated with this deficiency were dermatitis, alopecia, skin ulcerations, brown eye exudate, diarrhea, transverse cracking and bleeding of the feet, and inflammation of the mucous membrane of the mouth. There was not, however, a reduction in urinary biotin excretion (table 2), nor retardation of growth (fig. 3). The appetites of deficient animals were normal and comparable to those of litter-mate controls (table 3).

Cunha, Lindley and Ensminger ('46), in their studies with desiccated egg white, reported somewhat similar deficiency symptoms with pigs 12 to 22 weeks of age. These workers, starting with 8-week-old pigs, however, did not report skin ulceration and inflamed mucous membranes as characteristic

TABLE 3

Relationship of dietary intake to growth

(Trial I)

DAYS ON DIET	CONTROL			DEFICIENT					
	Pig 8			Pig 9			Pig 10		
	Period intake	Period gain	Intake per lb. gain	Period intake	Period gain	Intake per lb. gain	Period intake	Period gain	Intake per lb. gain
	liters	*lb.*	*liters*	*liters*	*lb.*	*liters*	*liters*	*lb.*	*liters*
7	2.934	0.75	3.91	2.421	0.50	4.84	3.011	1.00	3.01
14	4.246	1.25	3.40	3.746	0.75	4.99	4.265	1.50	2.84
21	6.240	2.25	2.77	4.724	1.75	2.70	6.230	2.25	2.77
28	9.360	3.50	2.67	6.720	1.75	3.84	9.360	3.00	3.12
35	13.690	3.50	3.91	9.330	3.00	3.11	13.654	4.25	3.21
42	15.720	4.25	3.70	11.600	3.00	3.87	15.463	3.50	4.42
49	19.350	5.00	3.87	15.037	3.75	4.01	18.915	4.25	4.45
56	26.974	7.25	3.72	15.433	3.25	4.75	20.119	4.25	4.73

(Trial II)

DAYS ON DIET	CONTROL			DEFICIENT								
	Pig 26			Pig 23			Pig 24			Pig 25		
	Period intake	Period gain	Intake per lb. gain	Period intake	Period gain	Intake per lb. gain	Period intake	Period gain	Intake per lb. gain	Period intake	Period gain	Intake per lb. gain
	liters	*lb.*	*liters*	*liters*	*lb.*	*liters*	*liters*	*lb.*	*liters*	*liters*	*lb.*	*liters*
7	3.640	0.50	7.28	3.640	0.50	7.28	3.070	0.50	6.14	3.445	0.50	6.89
14	7.380	1.75	4.22	7.410	2.25	3.29	6.960	1.75	3.98	7.070	2.00	3.54
21	10.815	3.00	3.61	10.910	2.75	3.97	10.940	2.50	4.38	10.960	3.00	3.65
28	16.400	4.50	3.64	16.710	4.75	3.52	13.910	5.00	2.78	16.140	4.25	3.80
35	26.225	9.00	2.91	24.380	8.50	2.87	19.585	5.25	3.73	24.450	6.25	3.91
42	34.650	4.50	7.70	28.835	4.25	6.78	28.465	6.25	4.55	30.400	6.00	5.07
49	39.900	10.00	3.99	33.990	7.00	4.86	34.530	8.75	3.95	36.620	8.75	4.19
56	42.000	7.00	6.00	33.680	6.50	5.18	35.210	9.25	3.81	37.410	8.00	4.68

symptoms. Figure 1 illustrates the syndrome associated with a biotin deficiency of the suckling pig fed a purified diet. Figure 2 shows its normal litter mate receiving 0.01 mg of biotin per kilogram of liquid diet.

The deficiency symptoms in the studies reported here followed a uniform course. Dermatitis first appeared on the ears, neck, top of the shoulders and tail and eventually spread

Fig. 1 Biotin-deficient pig 9. Note severe cracks on feet and ulcers on ham.

over the animal's entire body. The dermatitis was followed by general alopecia and finally ulceration of the hams and bellies.

Transverse cracking and subsequent bleeding of the soles and top of the hoof head gradually became apparent. This abnormal condition increased in severity as the experiments progressed until the animals could walk only with difficulty and pain.

Inflammation of the mucous membranes of the mouth, while very pronounced, was not associated with loss of appetite

or difficulty in sucking and thus was apparently not of a painful nature.

Dark brown eye exudate and diarrhea, generally associated with most water-soluble vitamin deficiencies, occurred at an early date. The diarrhea was easily alleviated by feeding sulfathalidine (table 2).

Deficient animals in both trials weighed approximately the same as controls (fig. 3) and post mortem studies revealed no

Fig. 2 Litter-mate control pig received adequate amount of biotin.

macroscopic abnormalities. The control animals did not develop any of the above symptoms and were apparently normal in every respect from the standpoint of gross pathology and macroscopic observation.

Trial I

Deficiency symptoms were more severe in pigs 9 and 10 in trial I than in pigs 23, 24, and 25 in trial II.

The urinary biotin excretion of the control pig showed the expected increase with increased intake (table 2). The deficient pigs, on the other hand, failed to show the expected decrease of biotin excretion and continued to excrete the vitamin, although at a reduced rate. Since the symptoms of biotin deficiency were apparent at this time (29 days), sulfathalidine was added to the diet at the rate of 0.5 gm per feeding (3 gm per day), and supplementation was continued

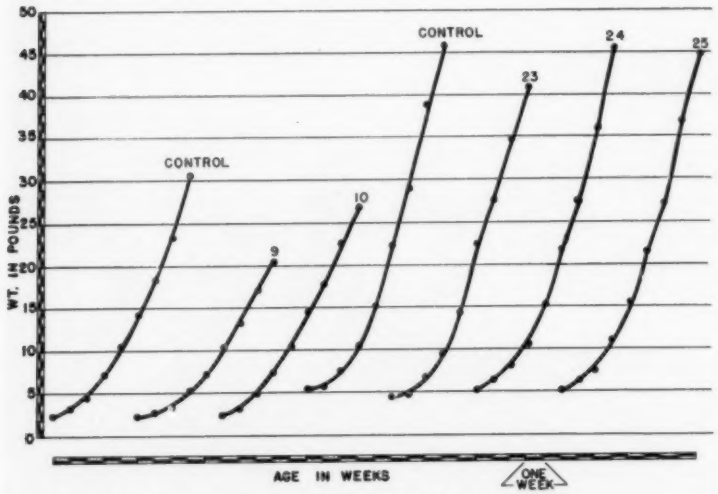

Fig. 3 Growth of pigs on biotin-deficient diet.

for 20 days. It is apparent in table 2 that the animals continued to excrete biotin in spite of this intake of sulfathalidine.

Further investigation showed that the urine contained an unknown ether-soluble material which stimulated the growth of the microorganism used in the microbiological assay for biotin. Consequently, the urine samples obtained from the pigs in trial II were extracted with ether before being assayed for biotin. The results obtained in trial II for the

urinary excretion of biotin give a truer indication of biotin excretion under the conditions of this experiment.

From the 46th day through the final day of the experiment (56th day), 10 μg of biotin were fed daily to each deficient animal. Neither pig showed any great response to this supplementation, nor was there any increase in urinary biotin excretion during this period of supplementation.

Trial II

Pigs on this trial followed the same general pattern as the pigs on trial I in developing biotin-deficient symptoms, although the symptoms were not so severe.

Sulfathalidine feeding at the rate of 6 gm per day (1 gm per feeding) was initiated on the 42nd day of the experiment and continued until termination on the 56th day in an attempt to inhibit apparent intestinal synthesis of biotin (table 2). Again this supplementation failed to cause great reduction in biotin excretion or any changes in deficiency symptoms. This may have been due either to the fact that the sulfathalidine did not inhibit the organisms responsible for intestinal synthesis of biotin or perhaps some synthesis of biotin was occurring in the tissues.

The fact that the biotin-deficient animals in both trials grew at a rate comparable to that of their litter-mate controls would indicate that the pigs were obtaining some biotin from either intestinal or tissue synthesis. The amount of biotin the pigs obtained from either of these sources, while sufficient for growth, was not, however, enough to prevent the physical symptoms observed.

SUMMARY

In suckling pigs started on experiment when the pigs were 48 hours old, biotin deficiency was apparently associated with ulceration of the skin, transverse cracking of the soles and top of the hoof, inflammation of the mucous membranes of the mouth, dermatitis, and alopecia. The urinary excretion

of biotin failed to show the expected decrease and was not materially affected by the feeding of sulfathalidine. This may indicate that sulfathalidine does not inhibit the organisms responsible for intestinal synthesis of biotin in the suckling pig, or perhaps that some synthesis of biotin occurs in the tissues.

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PRIMATE NUTRITION

I. THE CEBUS MONKEY — NORMAL VALUES [1]

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FOUR FIGURES

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In the past three years the nutritional requirements of and the manifestations of specific nutritional deficiencies in the Cebus monkey have been studied in this laboratory. This genus of New World monkey was introduced as an experimental animal in nutritional studies by Shaw ('49), who described the manifestations of vitamin C deficiency.

The need for further investigation of primate nutrition is widely recognized. The Old World macaque monkey (genus Macaca and Mulatta) has been the most widely used subhuman primate in experimental studies, but these animals are subject to several practical disadvantages. They must be imported from great distances and the consequent crowding and inadequate care lead to gross trauma and high mortality, especially among the young animals which are most useful as experimental subjects. They are particularly susceptible to tuberculosis, with consequent hazard to both the experiment and the operator. Experience with a large number of *Macaca rhesus* monkeys imported from India indicates a minimum prevalence rate of 5 to 10% of pulmonary tuberculosis (Chase, '50). The macaque monkey is unusually

[1] Supported in part by grants-in-aid from the National Heart Institute of the National Institutes of Health, Public Health Service; National Vitamin Foundation, Inc., New York, N. Y.; Swift and Company, Chicago, Ill.; and the Nutrition Foundation, Inc., New York, N. Y.

susceptible to dysenteric diseases, which appear to be endemic, so that the additional stress of either shipping or the experimental procedure leads to an excessive mortality which cannot be adequately controlled. The animals are active and require large cages, thus limiting the number of subjects which can be studied. They are, in addition, strong and agile and often require general anesthesia for examination — an objectionable procedure during an experimental period.

Cebus monkeys are readily available by air freight from South America. They are unusually hardy in respect to handling losses and freedom from both tuberculosis and dysenteric diseases. Our experience with 71 young adults, and that of Shaw ('50) with 20 animals, have revealed no tuberculosis either by tuberculin testing or at autopsy. The animals are small, weighing 800 to 1,500 gm when purchased, and rarely exceeding an adult weight of 2,000 gm. They can be adequately housed in metal cages measuring $19 \times 19 \times 14$ inches, which in tiers will allow facilities for 6 animals in approximately the same cubic space required by three macaque monkeys. Although the Cebus monkey can be made docile with training, their small size allows handling with gloves. We have devised a restraining board in which an animal can be held without anesthesia (fig. 1).

A disadvantage of the Cebus monkey is the small growth potential remaining when the animals are received. Although — as with the macaque — there is no certain means of determining exact age, judging from the stage of dentition and development of genital organs, the animals used are presumed to be young adults — probably one to two years of age. At this age growth is slow and an insensitive indicator of experimental conditions.

The taxonomical classification of Cebus monkey is difficult. Various shipments of monkeys from different areas of tropical South America indicate, by differences of coloration and conformation, that there are many variations of the genus. These variations comprise possible hybrids of many types. Although the animals used to appear to resemble either the

mammalogists' *C. capucinus* or *C. fatuella*, it is difficult to make a certain classification. Among animal men and zoo-keepers they are known collectively as "ringtails."

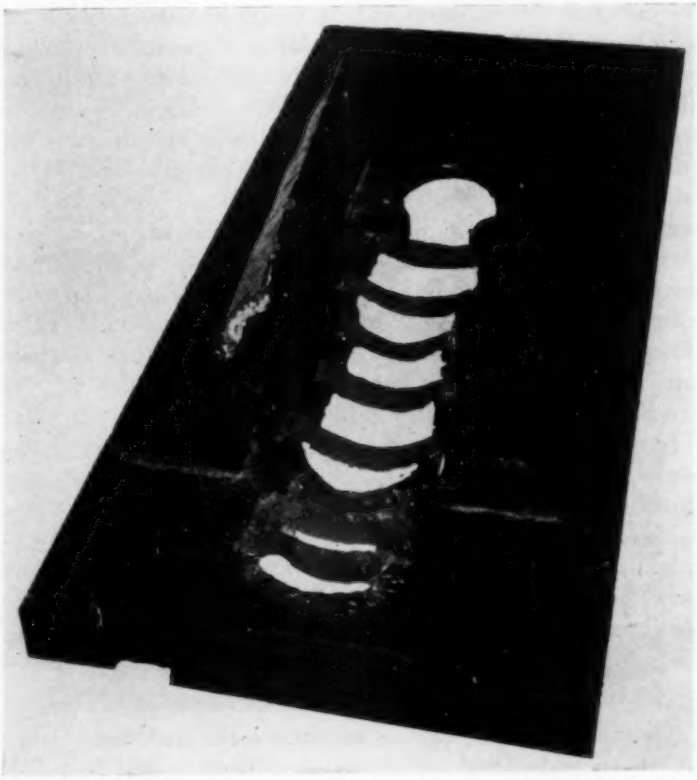

Fig. 1 A device for the restraint of unanesthetized monkeys during examination. The animal is held in a supine position on the web straps until the hinged cover can be closed. The head and all extremities are then free for procedures.

The animals have always been found free of ectoparasites. Occasionally a fungal dermatitis has been seen, which in the normal animals remains stationary. Intestinal nematodes have not been found. In an occasional animal an unidentified

species of *Pentastomada* is found in the cecum. *Hymenolepsis nana* is found frequently in the intestine. The most prevalent metazoic parasite has been the filaria, tentatively *Acanthocheilonema perstans*. Almost 100% of the animals are infected. Microfilaria can be easily demonstrated in the peripheral blood and with no evidence of periodicity. Adult worms are regularly found embedded in the mesenteric root, with bodies free in the peritoneum and occasionally encysted beneath the visceral pleura. Treatment of a few animals with 1-diethylcarbamyl-4-methyl-piperazine hydrochloride (Hetrazan) has not been successful (Hewitt et al., '48). Periodic enumeration of the microfilaria in the blood over a three-year period has not indicated a significant change in their prevalence, either in relation to time or the dietary deficiency induced. It has not been possible to demonstrate a deleterious effect of these parasites. A positive correlation has been found between the number of blood eosinophiles and the number of microfilaria in the blood.

It is apparent that, with these variables, experimentation can best be controlled by the use of appropriate fore-periods allowing each animal to act as its own control. These fore-periods allow further completion of growth, so that in most animals, growth was near completion and body weight was used only to distinguish maintenance as related to the experimental procedure.

EXPERIMENTAL

The animals were kept in an air-conditioned room held at 25.5°C. in individual metal cages on wire screen floors preventing access to the refuse tray (fig. 2). The food cups are designed to allow feeding without opening the cage. The small aperture through which the animal reaches the food minimizes spillage. Water is supplied with a fountain-type glass bottle fitted with a copper drinking spout. With this arrangement, as many as 36 animals may be housed in a room 8×15 feet.

Dietary procedures

New animals were tuberculin-tested with 0.00002 mg of tuberculin (purified protein derivative) intracutaneously and quarantined for 5 days. Food was withheld for 24 hours and

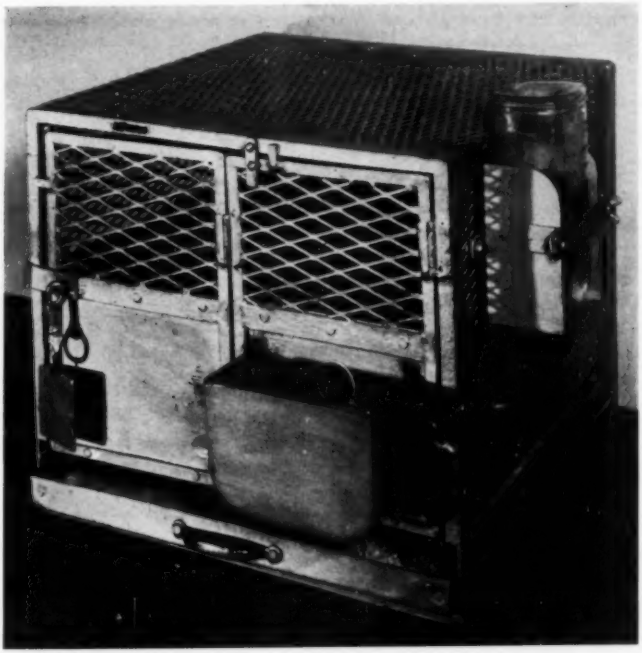

Fig. 2 A metal cage with a removable litter tray used for housing one monkey. Note that both food and water supplies are accessible without opening the cage. For balance studies the litter tray is replaced by a conical collection pan draining into a bottle. Six such cages are conveniently assembled on a metal rack with wheels.

they were then offered the complete purified diet, 850 (table 1). Choline chloride, inositol, and *p*-aminobenzoic acid were combined in the diet when it was prepared at weekly intervals. The diets were stored in tight cans at 0 to 5°C.

The remaining vitamins were combined in 25% ethanol as shown in table 2. This solution was made to contain the daily supply for each animal in a volume of 5 ml. The animals were fed once daily an amount of the basal diet, which they would

TABLE 1

The purified diet found adequate for a three-year period with Cebus monkeys

DIET 850	
COMPONENTS	AMOUNT
	%
Casein (vitamin-free)	25.0
Sucrose	60.6
Corn oil (Mazola)	8.0
Cod liver oil, U.S.P.	2.0
Salts IV [1]	4.0
p-Aminobenzoic acid	0.1
Inositol	0.1
Choline chloride	0.2

[1] Hegsted et al. (J. Biol. Chem., *138:* 459, 1941).

TABLE 2

The daily allowance of vitamins added to each monkey's ration [1]

VITAMINS	AMOUNT
	mg/5 ml
Thiamine HCl	1.0
Riboflavin	1.0
Pyridoxine HCl	1.0
Niacin	4.9
Ca pantothenate	3.0
Folic acid	0.1
Biotin	0.02
Ascorbic acid	25.0

[1] In 25% ethyl alcohol.

eat in 24 hours, and 5 ml of the vitamin mixture were added to each animal's portion and thoroughly mixed with a spatula. With this procedure, spillage does not exceed 10% in normal animals. Normal food consumption is 50 to 90 gm/day (180 cal./kg/day).

Growth performance

Representative weight increases on this regime are shown in figure 3. It has been established that the addition of cellu flour to diet 850 is not essential for maintenance of weight and good health. It was also found that neither growth nor

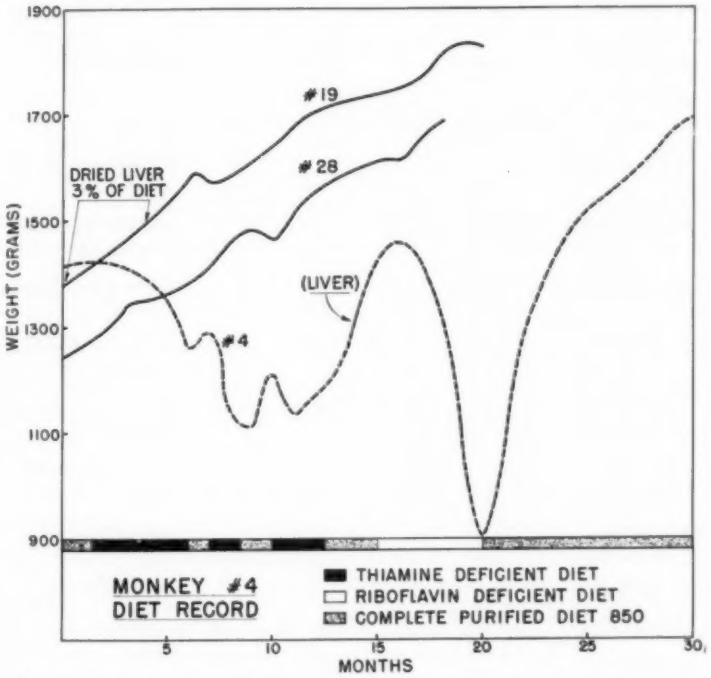

Fig. 3 The growth of Cebus monkeys on several diets. The liver supplements were defatted whole liver powder.

blood values were improved by the addition of fresh heart, fresh liver, or whole liver powder to diet 850. Animals have been maintained on these diets for three years and they remain in good health. In several instances animals have been made severely deficient, and with resumption of the complete diet they have regained good health (fig. 3, monkey 4).

TABLE 3

Normal chemical values for Cebus monkeys

	NUMBER DETERMINATION	ANIMALS	MEAN	S.D.	METHOD
Blood glucose (fasting) mg %	15	5	68.9	24.6	Nelson ('44)
Plasma cholesterol (total) mg %	24	6	92.5	31.5	Sperry ('38)
Plasma cholesterol (free) mg %	24	6	24.6	11.6	Sperry ('38)
Total plasma protein gm %	67	5	7.66	0.47	Pillemer and Hutchinson ('45)
Plasma albumin gm %	65	5	3.64	0.47	Pillemer and Hutchinson ('45)
Plasma globulin [1] gm %	65	5	3.50	0.67	Pillemer and Hutchinson ('45)
A/G			1.10	0.37	Calculated from Pillemer
N. P. N. mg %	14	6	34.1	10.0	Koch and McMeekin ('24)
Blood volume ml/100 gm B.W.	65	5	10.2	1.15	Wang and Hegsted ('49)
Plasma volume ml/100 gm B.W.	65	5	5.48	1.34	Calculated from Het.
Bromsulfalein (4 mg dose) 5 min.	63	5			Wang and Hegsted ('49)
15 min.			43.4	7.47	
% Clearance			81.3	8.21	
Total plasma riboflavin μg %	38	13	3.78	1.17	Burch et al. ('48)
Flavin adenine dinucleotide μg %	21	13	2.65	1.03	Burch et al. ('48)
Free riboflavin plus flavin mononucleotide μg %	21	13	1.15	0.36	Burch et al. ('48)

[1] Corrected for fibrinogen.

Normal values

In order to obtain a measurement of the degree of variation between animals, measurements of several physiological functions were made on "normal" animals maintained upon the complete purified diet for periods of up to three years. A summary of these values, which is believed partially to characterize this species, is presented in table 3, along with statistical perimeters of variation.

TABLE 4

Normal blood cell values for Cebus monkeys

	NUMBER DETERMINATIONS	ANIMALS	MEAN	S.D.	METHOD
R.B.C., million/mm³	118	20	6.98	0.79	Wintrobe ('46)
Hemoglobin, gm %	171	25	16.58	1.35	Alkaline hematin — Klett
Hematocrit, %	161	25	49.99	3.23	Van Allen ('24)
M.C.V., μ^3	118	20	72.2	7.31	Calculated
M.C.H., $\mu\mu$	118	20	23.8	1.83	Calculated
M.C.H.C., %	161	25	33.0	1.74	Calculated
Reticulocytes	36	9	0.45	0.34	Wintrobe ('46)
WBC, thousand/mm³	93	19	7.50	2.29	Wintrobe ('46)
PMN [1]	67	19	23.9	4.45	
Eosinophile [2]	67	19	6.84	6.18	
Basophile	67	19	0.60	0.95	
Lymphocytes	67	19	66.1	11.0	
Monocytes	67	19	2.64	2.79	

[1] Polymorphonuclear. Cell types are reported as per cent of total white blood cells.

[2] Eosinophile counts tend to be high in animals with heavy filarial infections. Since most animals were infected, this value is abnormally high.

The blood chemical determinations were not unusual. There was no tendency to obesity, despite the restriction of activity and ad libitum feeding.

The hematological data which have been obtained for characterization of the normal in this species are tabulated in table 4. It is apparent that the erythroid elements of the blood in this species are in somewhat higher concentration than in other primates reported (Hall, '29; Shukers et al., '38).

Experience has indicated that hematological measurements are often subnormal when animals are received in the laboratory. A period of about one month on the complete purified diet is necessary for stabilization at normal values. Neither supplements of natural foods (meat, milk) nor of whole liver concentrates appears to accelerate this hematopoiesis. Since animals of various degrees of maturity behave in the same fashion, it is believed that this is not a physiological change of blood values with maturity. New animals are routinely allowed this period of equilibration before experimentation is begun.

The blood smears indicated the small size of the red blood cells and a marked degree of anisocytosis. Smears stained with Wright's stain initially presented difficulty in differential white blood cell counting. The lymphocytes characteristically exhibited much variability in appearance, ranging from large, pale cells to the small, dense characteristic lymphocytes seen in smears of human blood. Peroxidase stains were done routinely for several months until some confidence in our ability to differentiate the lymphocytic series from the granulocytes and monocytes was reached. As in the rhesus monkey, the ratio of granulocytes to lymphocytes was much lower in the Cebus monkey as compared with human blood.

Animals were kept in single cages, so that no observations on heterosexual behavior have been made. On rare occasions a small bloody vaginal discharge was observed in females, suggesting menstruation. Recently Shaw has observed a successful pregnancy and survival of a single baby by a female maintained in captivity on a natural diet (Shaw, '50). Reproduction in captivity is reported to be rare in this species.

Electrocardiograms made with a string galvanometer machine especially adapted to small animals with a rapid heart rate and low intensity discharge (Rappaport and Rappaport, '43) are illustrated in figure 4. These tracings indicate a close similarity to human tracings with a large increase in heart rate.

The life span of these animals is not known. A single individual has been seen whose age, we believe, is authentically stated to be 26 years. Aside from worn teeth and a body weight of 2,500 gm, the animal did not appear greatly different from a young animal. Experienced animal men believe the life expectancy in the wild state to be 10 to 15 years. The animal mentioned above was an organ grinder's companion.

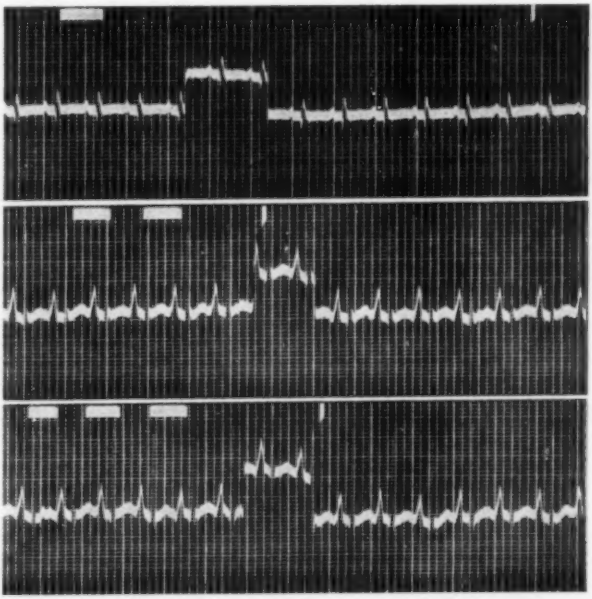

Fig. 4 Electrocardiographic tracings from a normal Cebus monkey. The film speed was 50 mm per second.

The quality which particularly recommends this species for nutritional research is their remarkable resistance to the stress of induced deficiency states. Deficient animals have been able to survive prolonged and severe nutritional illnesses as well as major surgical procedures with a resiliency far exceeding that of other animals studied in such circumstances.

SUMMARY

A description of the techniques for the laboratory care and management of a new primate species, the Cebus monkey, for experimental nutrition has been presented. Selected chemical and hematological measurements have been made which define a "normal" with its variations for this species. A purified diet and feeding technique are described which allow maintenance of good health for periods of at least three years.

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PRIMATE NUTRITION

II. RIBOFLAVIN DEFICIENCY IN THE CEBUS MONKEY AND ITS DIAGNOSIS[1]

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SIX FIGURES

(Received for publication December 19, 1951)

The manifestations of riboflavin deficiency are notably non-specific. In clinical medicine the diagnosis of deficiency is based upon such vague and variable clinical signs as cheilosis, magenta tongue and seborrheic dermatitis. A precise and reliable biochemical test would eliminate many of the difficulties encountered in evaluating the presence and severity of riboflavin deficiency, either in a single patient or in a population.

The studies reported here were made in an effort to adapt the chemical measurement of riboflavin and its derivatives in blood to this purpose. The first requirement for such a diagnostic method is a description of the correlation between the dietary intake and clinical manifestations of riboflavin deficiency and the blood levels of riboflavin. For this purpose riboflavin deficiency has been produced and studied in Cebus monkeys.

Riboflavin deficiency has been described previously in several species, including the rat (Wagner et al., '40), the dog

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(Street and Cowgill, '39; Sebrell and Onstott, '38) and the macaque monkey (Waisman, '44; Cooperman et al., '45). Some form of dermatitis is characteristic of the deficiency in all these species. Weight loss antecedes death in all except the adult dog, where the deficiency is often terminated by sudden death (Street and Cowgill, '39; Sebrell and Onstott, '38; Mann et al., '52a) without premonitory symptoms. Cooperman et al. ('45) produced riboflavin deficiency in young macaque monkeys and noted weight loss, a generalized dermatitis and a profound anemia. Although riboflavin supplements would rapidly correct the growth deficit and skin lesions, the anemia was not cured by this means. It was then concluded that the diet used was not complete for this species of primate. The authors estimated the daily requirements of the growing macaque to be 25 to 30 μg of riboflavin per kilogram of body weight.

Sebrell and Butler ('39), Williams et al. ('43), Keys et al. ('44) and, more recently, Horwitt et al. ('49) have described the effect of prolonged limitation of riboflavin intake upon adult human beings. Horwitt has described the irregularity of appearance of skin lesions and has emphasized the importance of predisposing trauma or abrasion in the etiology of skin signs. In none of these human experiments was anemia described. Horwitt estimates the riboflavin requirement of an inactive adult to be 1.1 to 1.6 mg per day, amounting to 20 μg per kilogram per day.

The interpretation and utility of blood riboflavin measurements have been complicated by the conflicting data obtained with various methods. Axelrod et al. ('41) reported no significant reduction in the blood riboflavin levels of patients believed riboflavin-deficient. These authors then concluded that such measurements were of no use. Repeated attempts with microbiological methods applied to whole blood have led to technical difficulties (Bradford and Coke, '45; Strong et al., '41). An advance was made when Burch et al. ('48) developed a photofluorometric method applicable with small amounts of serum. Serum analysis of 13 normal individuals

and a few subjects deprived of riboflavin for several days suggested to these authors that this method might profitably be applied to the problem of the diagnosis of ariboflavinosis.

METHODS

The experiments described have involved 14 monkeys studied in three experiments during a two-year period. The care and feeding of the animals have been described (Mann et al., '52b). Diet 850 and the vitamin supplements used are also described in the preceding paper.

The animals were weighed weekly or more often. Post-absorptive blood for analysis was obtained by venipuncture of the unanesthetized animals. The heparinized blood was used for hematological measurements and plasma was used for riboflavin measurements by the method of Burch et al. ('48) with minor modifications.

At death animals were autopsied and the organs weighed. Tissue fragments were fixed in neutral formalin and Zenker solution. These were then embedded in paraffin and stained by Mallory's technique.

Liver lipid analysis was carried out as follows: An aliquot of fresh liver was taken to a tared container for drying to constant weight at 106°C. Another suitable aliquot was ground in a mortar with three weights of anhydrous sodium sulfate. The triturate was dried at 50 to 60°C. and extracted for 24 hours in a Soxhlet apparatus with redistilled chloroform. The chloroform was removed and the residue taken up in petroleum ether and filtered through fat-free paper into a volumetric flask. Aliquots of this extract were analyzed for total lipid by gravimetry, phospholipid by the Fiske and Subbarow ('25) method for phosphorus, and cholesterol by the method of Sperry ('38). Values are expressed as per cent of wet liver.

Riboflavin supplements were given orally from a pipette in two divided daily doses unless otherwise indicated.

Analysis of diet 850 by chemical (Hodson and Norris, '39) and microbiological procedures indicated a riboflavin content

of 0.25 μg per gram of diet. Since the average daily food intake (determined by periodic measurement) of these animals was 40 to 60 gm, the animals received 10 to 15 μg of riboflavin per day from this source.

RESULTS

Experiment I

In a preliminary experiment two animals, M12A and M8A, were placed on diet 850 and diet 850 with 3% 1:20 liver

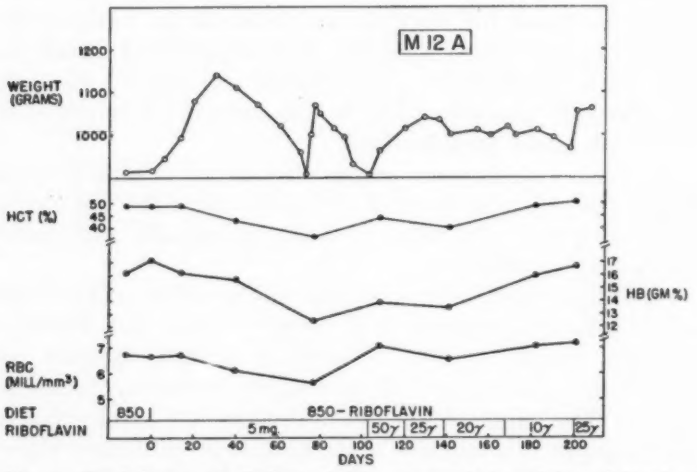

Fig. 1 The influence of riboflavin deficiency upon body weight and blood cell values of a Cebus monkey. The 5-mg supplement was given on the 72nd day.

extract,[2] respectively. Riboflavin was omitted from the vitamin supplement mixture. Animal M12A thus received 10 to 15 μg per day and M8A, 190 μg. Figures 1 and 2 illustrate the following points: Without the riboflavin furnished by the liver supplement, animal M12A lost weight steadily after 4 to 5 weeks. Graded doses of riboflavin given orally produced prompt growth responses, depending upon the amount given.

[2] Wilson's.

Animal M8A (fig. 2) was able to gain weight steadily on the liver-supplemented diet. When the liver concentrate was withdrawn, the animal's weight dropped steadily. Supplements of riboflavin produced dramatic weight gains in each animal and 1 mg of riboflavin daily returned animal M8A to apparent good health.

The deficiency produced was *not* associated with the development of anemia. Changes in the blood cell values suggested that supplementation with riboflavin, which produced rapid

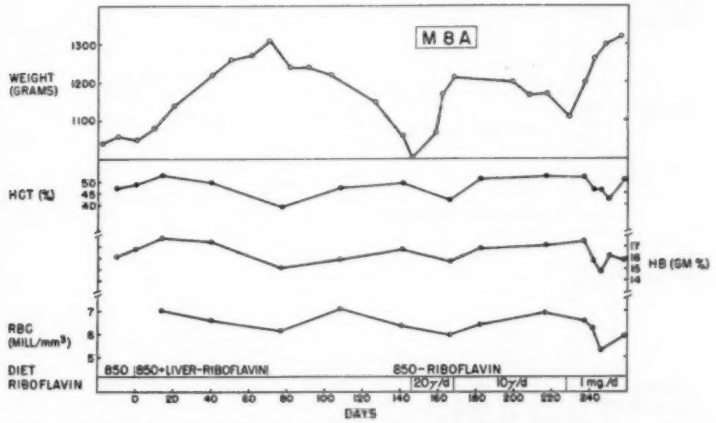

Fig. 2 The influence of the level of dietary riboflavin upon weight and blood cell values of a Cebus monkey.

growth, was associated with a fall of hemoglobin, hematocrit and red blood cell values; e.g., animal M8A on day 141 and those following and day 237 and those following.

No specific ocular or skin lesions were found in either animal. Animal M12A began the experiment with a minor fungal dermatitis and this became much more extensive during the deficiency, receding with riboflavin supplementation. Monkey M8A developed minor lesions on the ulnar area of the wrists after 65 days on the deficient diet. These lesions were diffuse, encrusted and seborrheic in appearance. They

were more extensive on the right hand and were believed to be initiated by rubbing on the cage aperture during eating. In both animals there was a diffuse loss of hair. No changes of the tongue or mucous membranes were observed. With treatment, new hair growth appeared as a lanugo-like coat and the skin assumed a soft texture and pink color suggesting regeneration.

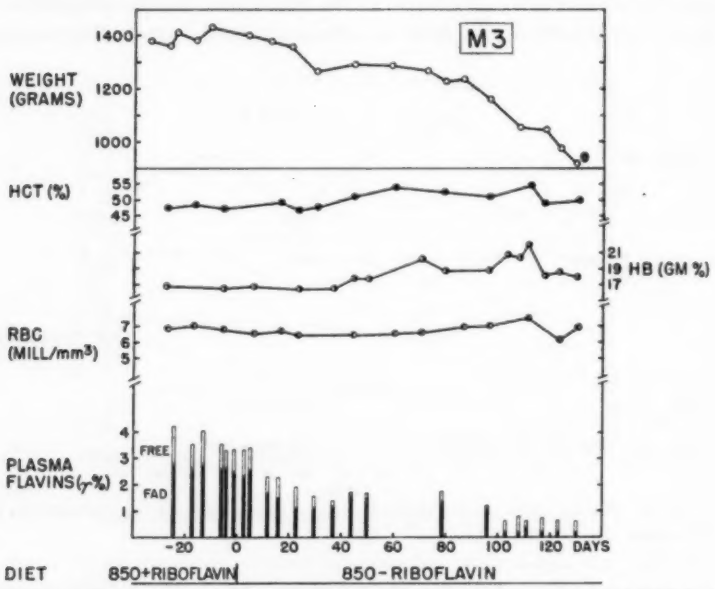

Fig. 3 The response of a Cebus monkey maintained on a riboflavin-deficient diet until death. The failure to develop anemia despite great weight loss and depletion of serum flavins is well-illustrated.

Experiment II

Four young adult animals were then placed on experiment with diet 850. In addition to body weight and hematological measurements, the levels of plasma free riboflavin, flavin-adenine-dinucleotide and total riboflavin were determined at intervals. After an initial control period with a complete

diet, riboflavin was omitted from the vitamin supplement mixture. Animal M8 of this experiment is the same as M8A of the preliminary experiment described above.

The course of representative animals in this second experiment is described in figures 3 and 4. These data indicate that serum total riboflavin is indeed a most sensitive indicator of riboflavin intake. Within 5 to 11 days after the reduction of riboflavin intake, the plasma flavin levels had decreased

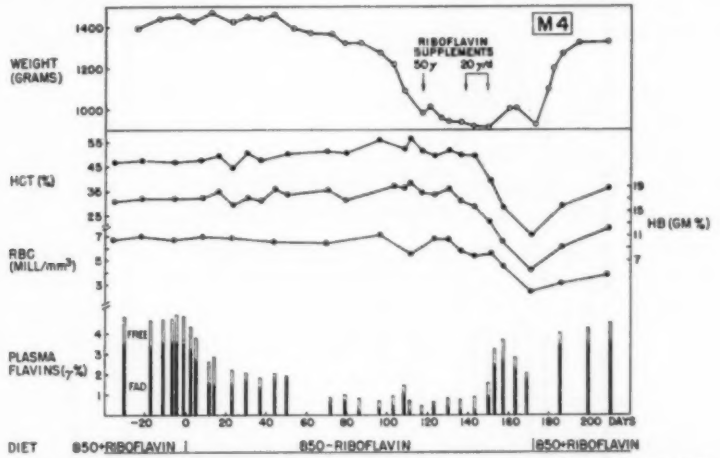

Fig. 4 The response of a Cebus monkey to severe riboflavin deficiency indicates the sensitivity of serum levels to intake of riboflavin and the paradoxical response of blood cell counts to both deficiency and riboflavin therapy.

significantly. The analysis of the mean serum values for the 4 monkeys is illustrated in table 1. While the serum free riboflavin at first seemed the most sensitive component, it did not prove the most reliable, due to the somewhat larger variability both physiologically and technically. These variations are reflected in the delayed appearance of significant differences between control and deficient levels of free riboflavin.

Animal M4 (fig. 4) was followed for 200 days. The data indicate a steady rise of red blood cells values until about

TABLE 1

The influence of riboflavin deficiency upon the plasma flavins of 4 Cebus monkeys. (The appearance of significant differences was determined by the application of a "t" test)

		PLASMA RIBOFLAVIN AS μG % ± STANDARD ERROR OF MEAN								
Day of deficiency	Number of observations	Total [1]		Significance of differences of means	FAD [2]		Significance of differences of means	Free and FMN [3]		Significance of differences of means
(Control)	26	3.58	0.183		2.58	0.173		1.03	0.070	
4	8	3.77	0.300	not sig.	2.59	0.131	no diff.	1.18	0.284	not sig.
11	8	2.29	0.170	$p < 0.01$	1.21	0.155	$p < 0.01$	1.08	0.208	not sig.
16	4	2.29	0.105	$p < 0.01$	1.50	0.050	$p < 0.01$	0.79	0.085	$p > 0.2$
23	4	1.70	0.115		0.76	0.080		0.94	0.120	p 0.05
30	4	1.37	0.135		0.82	0.045		0.58	0.230	
37	4	1.13	0.050		0.63	0.035		0.50	0.160	
79	4	1.01	0.070		0.55	0.095		0.46	0.065	
86	4	1.52	0.060		1.04	0.050		0.48	0.040	
102	4	0.55	0.080		0.20	0.010		0.35	0.070	
116	4	0.98	0.055		0.75	0.055		0.23	0.045	
129	4	0.55	0.120		0.29	0.010		0.26	0.040	
135	2	0.70	0.099		0.29	0.028		0.41	0.000	
Estimated standard deviation of duplicates		0.17						0.12		
Coefficient of variation		8.04						16.6		

[1] Total — total plasma riboflavin.
[2] FAD — flavin-adenine-dinucleotide.
[3] Free and FMN — free riboflavin plus flavin-mononucleotide.

the 100th day of deficiency. When riboflavin supplements were given on the 117th day and again on the 138 to 149th days, a pronounced fall of blood values associated with a gain in weight was noted. When the animal was returned to the complete diet 850 with riboflavin, rapid growth to normal levels ensued and was associated with a slow return of blood values toward normal. The failure of animals M3, M6 and M8 to develop a significant anemia until only very shortly before death, when anorexia developed also, emphasizes the unique influence of riboflavin deficiency on the hematopoietic system.

Non-specific skin lesions of varying severity developed in two of these animals. The encrusted seborrheic dermatitis was limited to the hands. Oral or ocular lesions were not observed. In all animals there was a diffuse loss of hair with a flaking, dandruff-like dryness of the skin. When deficient animals were treated, in addition to the rapid weight gain associated with a return of vigor and activity, the improvement of the texture and appearance of the skin and coat was the next most dramatic response.

Experiment III

A third experiment was then carried out using 8 normal, young adult monkeys. The growth responses of the 5 deficient animals were similar to those described for the first two experiments. Additional growth respenses of deficient animals to oral riboflavin supplementation were obtained. A graph relating growth in deficient animals to the daily supplement level is illustrated in figure 5. It appears that in this species 30 to 40 μg of riboflavin per kilogram of body weight per day lead to a maximum growth response of deficient animals.

The plasma riboflavin levels for the deficient and control animals in the third experiment are shown in figure 6. In this experiment the deficiency was produced with the use of a washed casein very low in riboflavin. After 35 days, when

the deficiency was well marked, the diet was prepared with the usual washed casein furnishing 0.22 μg of riboflavin per gram of diet. As in the previous experiment, serum levels of riboflavin tended to level off at a minimum concentration

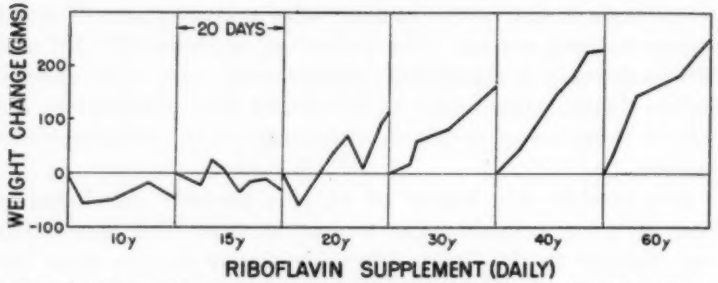

Fig. 5 The growth response of riboflavin-deficient monkeys to oral riboflavin supplementation.

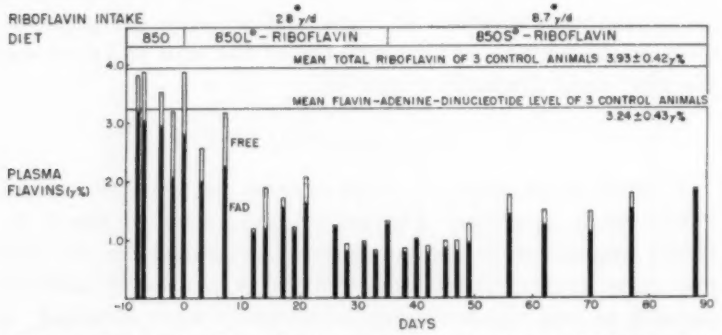

Fig. 6 The relationship of the plasma riboflavin levels of 5 deficient animals in experiment III to the intake of riboflavin. The diet 850L was diet 850 prepared with a casein furnishing 2.8 μg of riboflavin per day. Diet 850S was diet 850 prepared with a casein furnishing 8.7 μg of riboflavin per day.

which then persisted until death. When the diet change was made, supplying a small amount of riboflavin, the flavin-adenine-dinucleotide levels slowly increased. The serum riboflavin concentration was the most sensitive and the most reliable indicator of nutritional status.

The hematological changes in this experiment were not dramatic. However, a serious anemia did not develop. Table 2 indicates the blood values of the deficient animals according to the day of the deficiency. There was again a notable disproportion between the cachetic clinical state of the deficient animals and the mild degree of anemia. Although the three blood measurements were all significantly reduced during the first month of the experiment, it is apparent that the anemia was then self-limited and was no more severe at the 8th month

TABLE 2

The influence of prolonged and severe riboflavin deficiency upon the blood values of Cebus monkeys

PERIOD OF DEFICIENCY	NO. OF ANIMALS	NO. OF OBSERVATIONS	HEMOGLOBIN [1]		HEMATOCRIT [1]		RED CELL COUNT [1]	
months			*gm %*		*%*		*million/mm³*	
(Control)	8	16	17.2	0.39	51.3	0.65	7.38	0.16
1	5	15	15.9	0.38	49.2	0.91	6.83	0.26
2	5	13	14.7	0.39	46.3	1.14	6.23	0.21
3	4	12	15.0	0.41	47.5	1.03	6.27	0.25
4	4	15	14.5	0.38	46.9	1.32	6.32	0.25
5	3	9	14.3	0.47	44.8	1.80	6.38	0.33
6	3	12	14.3	0.37	46.8	1.75	5.91	0.22
7	3	9	15.4	0.39	49.8	1.13	6.29	0.31
8	3	9	14.9	0.36	47.7	0.98	6.58	0.27

[1] The mean values are followed by the standard errors. The values are all significantly reduced at the first month when tested with the "t" test. A pooled variance method was used and significance defined as a chance probability of 5% or less.

when two animals had died of the deficiency and the remaining three were in serious condition. The anemia was normocytic and normochromic. Although the white blood count tended to become elevated with the deficiency, there was no significant alteration of the differential count.

Supplementation with 1 mg of riboflavin per day required 30 to 40 days for the plasma levels to reach normality despite the rapid response in terms of clinical manifestations. The flavin-adenine-dinucleotide component was the last to reach normal levels, indicating again the sensitivity of this meas-

urement in revealing nutritional status. Clinical signs were variable and non-specific. Neither ocular nor oral lesions were observed. No instance of sudden death comparable to those seen in adult dogs was observed.

The analysis of liver lipids is described in table 3. The failure of these animals to develop fatty livers was confirmed by histological studies of the livers. There was no suggestion of the "yellow liver" syndrome described by Sebrell and Onstott ('38) in riboflavin-deficient dogs and in macaque monkeys by Cooperman et al. ('45). Although there was no significant increase of total lipids in the livers of riboflavin-deficient monkeys, there was a significant reduction of the

TABLE 3

The liver lipids in riboflavin-deficient monkeys compared with control animals (Mean values with standard errors are listed with the results of a "t" test of significance of differences of means)

NUMBER OF ANIMALS	TOTAL SOLIDS	TOTAL LIPID	TOTAL PHOSPHOLIPID	TOTAL CHOLESTEROL	FREE CHOLESTEROL
	gm %	*gm %*	*gm %*	*mg %*	*mg %*
Control 4	24.8 ± 0.41	5.13 ± 0.70	1.41 ± 0.20	404 ± 14	302 ± 10
Deficient 4	26.9 ± 1.47	3.47 ± 0.48	1.7 ± 0.26	225 ± 11	138 ± 21
"t"	2.40	1.83	0.62	4.00	4.30
"p"	> 0.05	> 0.10	> 0.50	< 0.01	< 0.01

liver cholesterol (table 3). This reduction is best explained as a response of the deficient animals to the stressful conditions of the experiment. We have demonstrated (Mann et al., unpublished data) that a general response to physiological stress is a reduction of serum cholesterol. We have also found that these serum changes are paralleled by similar changes in the liver and adrenal.

Histological examination of the tissue revealed 4 significant changes:

1. There was consistent evidence of demyelinization of peripheral nerves. In retrospect this finding may be responsible for the terminal ataxia observed. Monkey 4, which was cured of the deficiency, required several months to regain normal agility.

2. Epithelial atrophy was seen along the intestinal tract and in the bladder, with evidence of bacterial invasion and cicatrization. Sections of the sclerae did not reveal abnormalities.

3. The endocrine organs examined showed several consistent changes. In the two male animals the testicles showed hyperplasia of the interstitial cells. These cells formed extensive epithelial sheets, occupying a large proportion of the tissue substance. Spermatogenesis was not observed. The female animal showed pre-puberal ovaries. Since the exact ages and normal onset of puberty are not known, the lack of gonadogenesis cannot be evaluated.

The adrenals indicated cessation of the normal secretory cycle in two of the 5 animals autopsied. The thyroid glands were in all cases in the quiescent stage, with abundant colloid. The thymus glands were involuted.

4. The small arteries of the kidney and heart particularly showed medial fibrosis of a moderate degree, with intimal hyperplasia focally. The kidneys and spleens of two animals showed fibrotic changes suggesting endarteritis obliterans. No atherosclerosis was seen.

DISCUSSION

Our observations indicate the daily riboflavin requirement for maintenance of these animals to be 25 to 35 μg per kilogram of body weight per day. Deficiency of riboflavin was manifested by several non-specific changes: (1) loss of weight; (2) a variable seborrheic, scaling dermatitis which appears related to local trauma; and (3) loss of hair.

An immediate and profound fall of the plasma levels of riboflavin and its derivatives was consistently observed. Of these compounds, plasma flavin-adenine-dinucleotide appeared to be the most useful indicator of riboflavin deficiency.

Certain unusual hematological features were often associated with the development of riboflavin deficiency. A remarkable disproportion between the physical deterioration of the deficient animals and the maintenance of blood cellular

elements was frequently observed. Thus, deficient animals often were at the point of death before any evidences of anemia could be found.

In a few animals an increase of red blood cells and their contents seemed associated with a particular stage of the deficiency (e.g., fig. 2, 78 to 142 days; fig. 3, 20 to 110 days; fig. 4, 20 to 100 days). Studies continue with the hypothesis that a critical low level of tissue flavin concentrations acts through the resulting anoxia as a stimulus to red cell production. It has not yet been possible to produce a well-defined polycythemia by adjusting the riboflavin intake to produce this critical condition.

A similar trend toward polycythemia in riboflavin-deficient human subjects has been noted by Keys et al. ('44) and can be deduced from the data of Horwitt et al. ('49). When deficient animals were given riboflavin supplements leading to growth, a precipitous fall of blood values ensued, suggesting a competitive need for the same essential nutrient. The anemia which developed terminally in the monkeys in the present experiments can be explained by the onset of anorexia. The riboflavin-refractory anemia described in deficient macaque monkeys by Cooperman et al. ('45) was not observed in these experiments. While these contrasting experiences have been obtained with different primate species, nevertheless the differences are unexpected. An explanation may lie in differences between the feeding procedures used in the two laboratories. In the Wisconsin laboratories the daily vitamin supplements were fed separately in a dish. In the present work the vitamin supplements have been mixed with the day's food allotment. Since the food allotment was adjusted to the animal's appetite, and spillage by measurement did not exceed 10%, the vitamin intake was assured. A recent publication from the Wisconsin laboratories has described the cure of monkey anemia by the addition of supplementary levels of pteroylglutamic acid to the diet. The question then arises whether the anemia described following riboflavin and

other vitamin deficiencies has not resulted from a marginal intake of pteroylglutamic acid.

The skin changes in these animals were notably non-specific. The histological changes at autopsy were primarily limited to the endocrine system, where evidence of secretory activity was lacking in the thyroid and adrenal. The gonadal function appeared repressed, and in males there was a massive proliferation of the endocrine interstitial cells.

Contrary to previous reports, the liver was not fatty in deficient animals.

No great change of plasma flavins occurred after the riboflavin deficiency was well developed and the plasma content had reached minimum levels. Thus, this biochemical test, although allowing specific and sensitive indication of the onset and presence of deficiency, did not allow prediction of the final biochemical failure leading to death.

Anorexia was not a major sign of riboflavin deficiency until shortly before death. Ataxia was a prominent terminal symptom and is adequately explained by the peripheral nerve demyelinization seen at autopsy. Unlike the adult dog, these animals did not exhibit the dramatic "collapse syndrome." These young adult monkeys behaved like riboflavin-deficient adult rats in that after a large weight loss, anorexia and cachexia are followed by death.

SUMMARY

1. Riboflavin deficiency has been studied in the Cebus monkey.

2. Measurement of the plasma riboflavin and its derivatives has been found the most sensitive and specific indicator of deficiency.

3. The deficiency is not associated with pronounced anemia unless growth is stimulated with riboflavin, after which an anemia develops as rapid growth ensues. The maintenance of a normal or nearly normal cell pattern during severe deficiency suggests the possibility of an anoxic stimulus to blood formation. Neither fatty livers nor the collapse syn-

drome were seen. A significant diminution in liver cholesterol is related to the stressful influence of the deficiency.

4. The endocrine tissues studied, notably the gonads and adrenals, are profoundly influenced by riboflavin deficiency.

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GROWTH AND METABOLISM STUDIES WITH RATS FED RATIONS CONTAINING PURIFIED AMINO ACIDS [1]

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TWO FIGURES

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The amino acid requirements of the rat have been studied extensively by Rose and his co-workers. In a series of papers (Rose, '38; Rose and Womack, '46; Womack and Rose, '46; Borman et al., '46; Womack and Rose, '47; Rose, Oesterling and Womack, '48) they reported various interrelationships that exist between components of an amino acid mixture. They have further found that although rats will grow when fed a ration containing the 10 essential amino acids, an increase in the rate of growth results upon the addition of 9 of the non-essential amino acids. Ramasarma, Henderson and Elvehjem ('49) have similarly demonstrated a need for the non-essential amino acids for optimum growth of the rat. It should be noted, however, that even though an improvement in the rate of growth of rats was obtained upon the

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addition of the non-essential amino acids, the total weight gain was still inferior to that obtained with a ration containing casein as the nitrogen source.

Much work has been done in an attempt to determine the causes for the differences in growth noted between animals receiving free amino acids and those receiving casein as the principal source of nitrogen in the ration. Woolley ('45, '46a, b, '48) reported that the growth rate of mice receiving amino acid rations was significantly increased if small amounts of materials rich in strepogenin were added to the diet. However, Maddy and Elvehjem ('49) found that a ration containing 16 amino acids as the sole source of nitrogen gave essentially as good growth in mice as that obtained with intact casein, and that the addition of intact proteins that are rich sources of strepogenin failed to improve the rate of growth obtained on the amino acid diet. Ramasarma, Henderson and Elvehjem ('49) similarly found that the growth rate of rats fed acid-hydrolyzed casein, supplemented with intact casein, was not significantly better than that obtained with the unsupplemented ration.

This paper deals with studies made in an attempt to formulate a ration containing free amino acids as the principal source of nitrogen that would produce growth rates in weanling rats approximating those obtainable with an optimum amount of a protein such as casein. Furthermore, a more complete study has been made of the metabolic fate of ingested nitrogen and of the energy arising from a ration containing 19 amino acids as compared with a ration containing the same levels of nitrogen and energy derived from a ration containing intact casein.

EXPERIMENTAL

Weanling male albino rats of the Wistar strain were used in all experiments. The animals were placed in individual wire cages with raised screen flooring. Except for the body balance studies, food and water were supplied ad libitum and the weekly food consumption was determined. The

weights of the animals were recorded twice weekly and the experimental periods were of 28 days' duration. When the body balance study was made, the animals were pair-fed, water was supplied ad libitum, and the experimental period was of 56 days' duration.

The rations used had the following basic percentage composition: corn oil 5,[3] salts IV (Lichstein et al., '46) 4, monosodium phosphate monohydrate [4] 0.63, vitamin mixture 0.5, choline chloride 0.3, amino acid mixture or casein [5] as indicated, and sucrose to make up to 100%. Any further alterations or additions to these rations were made at the expense of sucrose. The 0.5 gm of the vitamin mixture in a sucrose base contained: thiamine hydrochloride 0.3 mg, niacin 1.5 mg, calcium-D-pantothenate 2.0 mg, inositol 100 mg, sodium para-aminobenzoate 100 mg, biotin 10 μg, pteroylglutamic acid 25 μg, 2-methyl 1,4-naphthoquinone 0.36 mg.

The compositions of the amino acid mixtures (AAM) are given in table 1.[6]

In compounding the rations, the amino acids were accurately weighed and thoroughly mixed and ground in a mortar until the entire mixture could be passed through a 14-mesh sieve. The remaining components were added to these mixtures, and the complete rations were then mixed in a large Hobart mixer.

In cases where it was necessary to add racemic amino acids, of which the D forms were unavailable, the total nitrogen supplied by these amino acids was used in the calculation of the nitrogen content of the rations. Although this is not an

[3] Supplemented with sufficient oleum percomorphum to supply 1666 I.U. vitamin A, and 236 I.U. vitamin D per 100 gm of ration. Also contains sufficient quantities of a mixture of crude tocopherols to furnish 6.0 mg alpha-tocopherol per 100 gm of ration.

[4] To supply the phosphorus contained in an 18% casein ration for which the salt mixture was designed.

[5] Vitamin-test casein, purchased from the Nutritional Biochemicals Corporation, was used throughout these experiments.

[6] The amino acids used in these studies were purchased from the Nutritional Biochemical Corp.

entirely valid procedure, it is believed to be more nearly correct than to disregard completely this added nitrogen. Correspondingly higher amounts of lysine, histidine and arginine were fed when provided in the form of the monohydrate or monohydrochloride.

TABLE 1

Composition of the amino acid mixtures

AMINO ACID	AMINO ACID MIXTURES (AAM)		
	I	IV	VI
L-lysine monohydrochloride	1.24	1.50	2.00
DL-tryptophan	0.40	0.40	0.53
L-histidine monohydrochloride	0.49	0.86	1.15
DL-phenylalanine	0.90	1.20	1.60
L-leucine	0.80	1.20	1.60
DL-isoleucine	1.00	1.82	2.42
DL-threonine	1.00	1.43	1.90
DL-methionine	0.60	0.80	1.07
DL-valine	1.40	2.00	2.66
L-arginine monohydrochloride	1.00	0.50	0.67
DL-serine	0.50	0.20	0.27
DL-alanine	0.40	0.40	0.53
L-tyrosine	1.00	0.60	0.80
L-asparagine	1.00	0.20	0.27
L-glutamic acid	4.52	2.00	2.66
Glycine	2.00	0.10	0.13
L-proline	0.20	0.20	0.27
L-hydroxyproline	0.10	0.10	0.13
L-cystine	0.20	0.20	0.27
$NaHCO_3$		1.27	1.69
% AAM in diet	19.38	17.61	23.25
% Nitrogen in diet	2.50	1.96	2.61

A control group, receiving a ration containing casein as the source of nitrogen, was included in each series. The rate of growth of the rats receiving the casein ration was assigned a value of 100%, and growth rates obtained on all other rations within the series were calculated as a percentage of the growth obtained with casein.

Although the number of animals per group was small, in all cases where definite results were obtained, the work was repeated at least once before any definite conclusions were drawn.

RESULTS AND DISCUSSION

Growth studies

The first investigation as to the growth-promoting properties of amino acid mixtures was made using a ration patterned after that of Maddy and Elvehjem ('49) for the mouse. This ration was found to be very unsatisfactory for promoting the growth of the rat. A total weight gain for 28 days of only 46 gm was obtained. When the ration of Ramasarma et al. ('49) was used, a gain of 59 gm was obtained. These weight gains are 29 and 37%, respectively, of that of the casein control group. Since neither of these mixtures was satisfactory for rapid growth of the Wistar strain of rat, a third mixture (AAM–I) was formulated. The results obtained with rats fed diets containing this mixture of amino acids as the principal nitrogen source are given in table 2. As can be seen in this table, the new mixture of amino acids also failed to support a satisfactory rate of gain for rats. Furthermore, supplementation of this ration with 1% monosodium glutamate did not increase the weight gains. The addition of 3.5 μg % vitamin B_{12} to this monosodium glutamate-supplemented ration increased the growth to 85 gm, or 54% of that obtained with the control group. The addition of 6.5% glutamic acid to the vitamin B_{12}, monosodium glutamate-supplemented ration decreased the total weight gain to 71 gm for 28 days. In contrast to the work of Smith and Elvehjem ('50) with the mouse, monosodium glutamate had no beneficial effects on growth or food consumption for the rat.

Since none of these amino acid rations was capable of supporting a good rate of growth with the rat, even with the addition of vitamin B_{12}, the optimum amino acid mixture used in Rose's laboratory (Rose, Oesterling and Womack, '48) was tested for growth promotion in the Wistar strain

of rat. This combination of amino acids (table 1, AAM-IV) differs from any previously reported here in that the levels of all the essential amino acids, with the exception of arginine, are higher and the levels of the non-essential amino acids are lower. However, the total concentration of amino acids in AAM-IV is lower than those of the first three mixtures studied. Thus the proportion of essential to non-essential amino acids is considerably altered.

As is shown in table 2, the growth obtained with this amino acid mixture was greater than any previously obtained, regardless of the presence of vitamin B_{12}. However, the addition of 0.4% 1:20 liver powder [7] or 3.5 μg % vitamin B_{12} increased the average weight gain from 95 to 120 gm. Rose has routinely added this liver powder at a level of 0.4% to amino acid rations. From these results it may be concluded that the active stimulatory principal in the liver powder is vitamin B_{12}. No further benefit was derived from increasing the level of the vitamin B_{12} in the ration.

Although an improvement in growth is obtained by using amino acid mixture AAM-IV supplemented with either vitamin B_{12} or liver powder the total gain is still only about 76% of that obtained with rats receiving the same level of nitrogen derived from intact casein. There are several possible explanations for this difference. Either the casein contains some additional growth factor, such as strepogenin, that is not present in the amino acid mixture, or the levels of some or possibly all of the amino acids in the mixture are limiting, or the individual amino acids are not present in optimum proportions.

When a ration containing AAM-IV as the principal nitrogen source was supplemented with a crude animal protein factor preparation,[8] the results obtained were very erratic. In one series of tests the animals receiving this supplement grew at a rate 86% of that of the controls receiving the casein ration, while in subsequent experiments the growth was only

[7] Wilson's.

[8] Pfizer's BiCon APF-3 +.

TABLE 2

Growth response and efficiency of food utilization of rats fed various diets

SUPPLEMENTS AND NITROGEN COMPONENT ADDED TO BASAL MIXTURE	NUMBER OF ANIMALS	NITROGEN IN DIET	AVERAGE WEIGHT GAIN FOR 28 DAYS	% OF GROWTH OBTAINED ON CASEIN DIET	FOOD EFFICIENCY [1]
		%	*gm*		
AAM–I	4	2.50	48	30.4	0.26
AAM–IV	3	1.96	95	60.7	0.36
AAM–IV + 0.4% liver [2]	3	1.96	121	77.1	0.40
AAM–IV + 3.5 μg % B_{12} [3]	3	1.96	120	76.4	0.40
AAM–IV + 7.0 μg % B_{12} + 10 mg % aureomycin HCl	3	1.96	132	81.4	0.38
AAM–IV + 7.0 μg % B_{12} + 10 mg % streptomycin SO_4 + 4.5% casein	3	2.61	136	84.3	0.47
AAM–VI + 7.0 μg % B_{12} + 10 mg % streptomycin SO_4	3	2.61	130	80.2	0.42
18% Casein	3	2.61	168	104.0	0.48
18% Casein + 0.5% APF [4]	3	2.61	162	100.0	0.49
18% Casein + 1.0% APF	3	2.61	159	99.0	0.47
18% Casein + 2.0% APF	3	2.61	166	103.0	0.48
18% Casein + 7.0 μg % B_{12} + 10 mg % streptomycin SO_4	3	2.61	167	104.0	0.48

[1] Food efficiency represents the gain in body weight in grams per gram of food consumed.

[2] Wilson's 1:20 liver powder.

[3] Pfizer's vitamin B_{12} oral grade solids.

[4] Pfizer's BiCon APF-3 +.

72 and 73% of that of the control groups. Therefore any beneficial effects resulting from the addition of APF are due to the presence of vitamin B_{12} in the supplement. An increase in the level of the APF supplement to 1 and 2% of the ration resulted in weight gains of 69 and 74%, respectively, of that of the control group. Thus, in the majority of cases the benefit derived from the addition of this supplement to the amino acid ration was comparable to that obtained by the addition of vitamin B_{12} alone. Furthermore, when a ration containing this same amino acid mixture and 3.5 μg % of vitamin B_{12} was supplemented with either streptomycin or aureomycin at levels of 4.0 mg % of the ration, no further increase in the weight gains of the rats was noted.

Increasing the level of vitamin B_{12} to 7 μg % and of aureomycin to 10 mg % of the ration resulted in an improvement in the growth of the animals. These results are presented in table 2, and they indicate that for rats receiving a ration containing free amino acids as the principal source of nitrogen, the optimum level of aureomycin is greater than 4 mg % of the ration.

Since it is possible that the levels of some of the amino acids may have been limiting, an amino acid mixture containing increased quantities of all 19 amino acids was compounded. The composition of this mixture is given in table 1, where the mixture is desginated as AAM-VI. In it the level of each amino acid was increased proportionately so that the ratios of the components to each other remained constant. A slight improvement in the growth of the rats was noted when this mixture, supplemented with 0.5% APF, was fed. However, when a ration containing AAM-VI supplemented with 7 μg % of vitamin B_{12} and 10 mg % of streptomycin SO_4 was fed to rats, a weight gain comparable to that obtained with a similar ration containing AAM-IV and the same levels of streptomycin and vitamin B_{12} was obtained. Therefore, in the presence of adequate quantities of either streptomycin or aureomycin, the addition of increased quantities of all the amino acids to AAM-IV was of no benefit.

Thus, nitrogen does not appear to be the limiting factor in this ration.

No further benefit was derived from the addition of intact casein at a level of 4.5% to either of these amino acid rations when supplemented with vitamin B_{12} and streptomycin (table 2). Even though casein will stimulate the growth of rats receiving amino acid rations, the same degree of stimulation can be obtained by the addition of either streptomycin or aureomycin at levels of 10 mg % of the ration.

It is possible that the addition of antibiotics to this type of ration stimulates the intestinal synthesis of necessary factors that are contained preformed in the casein. It is also possible that the antibiotics provide a more favorable medium in the intestinal tract for the utilization, by the host, of the ingested free amino acids. This may occur by the antibiotic's exerting its effects through a reduction of the total numbers of undesirable bacteria, resulting in a more healthy animal. This is in line with the original idea of Metchnikoff, who suggested that certain types of bacteria in the intestinal tract may be deleterious, either because they destroy certain vitamins or amino acids, or because they produce toxins that are detrimental to health. The work reported here is insufficient to permit any definite conclusions to be drawn as to the role that either the intestinal microorganisms or the antibiotics play in this increased growth of rats fed amino acid rations. Regardless of the cause of this growth stimulation, it can be definitely stated that strepogenin, per se, is not required in the diet of the rat.

Since a ration containing AAM-IV, supplemented with 7 μg % of vitamin B_{12} and 10 mg % aureomycin, supported a better rate of growth in rats than any amino acid ration tested thus far, it is of interest to analyze the differences in growth among this ration, the control ration containing casein, and a ration containing AAM-IV and the same level of vitamin B_{12} with no added antibiotic. The growth curves obtained with rats fed these three rations are presented in figure 1. As can be seen, a very slow rate of growth was

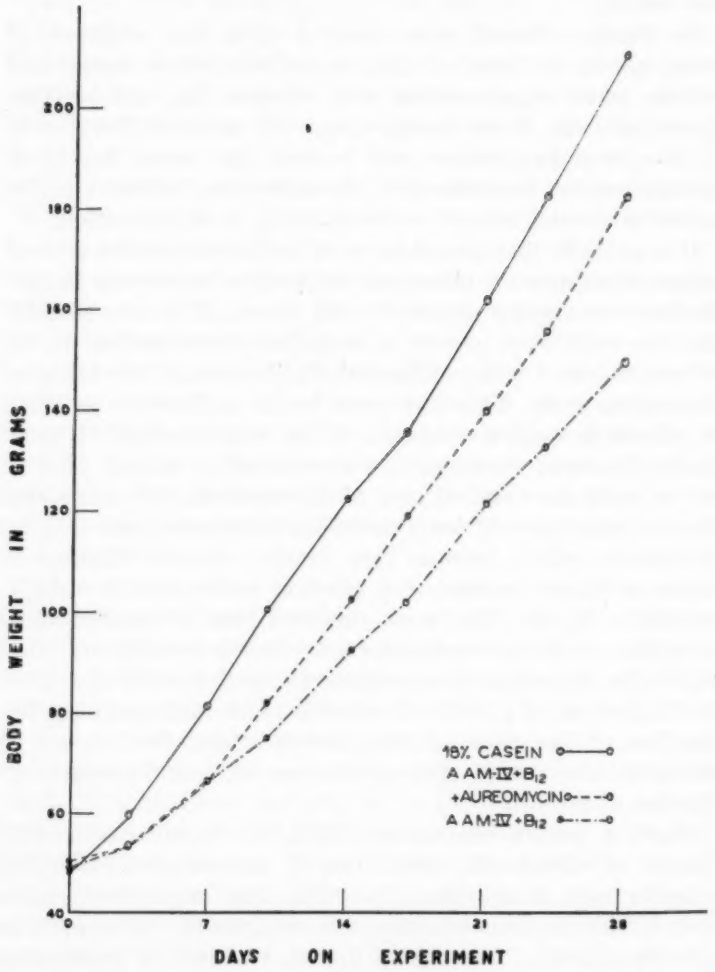

Fig. 1 Comparative growth of rats fed ad libitum rations containing casein, 19 amino acids plus vitamin B_{12}, or 19 amino acids plus vitamin B_{12} and aureomycin HCl.

obtained for the first week with rats receiving the free amino acid rations. From the 7th to the 28th day, the rats receiving the ration containing AAM-IV plus vitamin B_{12} and aureomycin grew at nearly the same rate as did the control group. The rats receiving the amino acid ration supplemented with vitamin B_{12} but with no added antibiotic increased their rate of gain after the first 10 days on experiment but continued to grow at a slower rate than did the other two groups of animals.

Thus it has been shown that even though this amino acid ration, supplemented with vitamin B_{12} and aureomycin, is capable of supporting a good rate of gain, a considerable period of time is required for the animals to adapt themselves to this type of ration. Further work has shown that this initial growth retardation occurs with rats weighing approximately 200 gm as well as with weanling rats. Such a phenomenon as this is rather difficult to evaluate. Since the total weight gains of rats receiving the amino acid ration were less than those of the casein controls, one would immediately assume that the more highly purified ration is incapable of supporting optimum growth. However, if the results obtained from only the second to the 5th weeks are compared, the true differences between the two groups are of doubtful significance. Nevertheless, the question of the physiological adaptability and palatability of a ration for animals must always be considered, since a diet is of no value if it is not consumed. Thus it must be concluded that all the amino acid rations tested so far are incapable of supporting as great a weight gain per unit of time as that obtained with the casein control diet.

In all studies reported, the casein control ration contained 0.5% APF. This supplement was added routinely and no investigation had been made to determine if any benefit was derived from the addition of this material. However, since it had been found that the addition of APF or antibiotics and vitamin B_{12} to amino acid rations would significantly increase the weight gains of rats, it was of interest to determine if these

three supplements would increase the rate of growth of rats fed a ration containing intact casein as the nitrogen source.

It was found that the addition to the control ration of either APF, at levels of 0.5, 1.0 or 2.0% of the ration, or a combination of vitamin B_{12} and streptomycin, failed to support a more rapid rate of gain than that obtained with the unsupplemented casein control diet. These data are presented in table 2.

The "vitamin-free" casein used in these studies was subsequently found to contain approximately 0.02 μg of vitamin B_{12} per gram. Therefore it is entirely possible that there was an adequate amount of this factor present in the casein, and that a further addition was without benefit.

Since the vitamin B_{12} content of purified proteins such as casein can vary from lot to lot, it would seem advisable to analyze test rations for their actual vitamin B_{12} content before any definite conclusions are drawn as to the effects of supplementing them with this vitamin. The present literature contains many conflicting reports pertaining to the value of vitamin B_{12} and the antibiotics in the nutrition of the rat. It is an established fact that vitamin B_{12} is essential for optimum growth and hematopoiesis in the rat; however, in many cases, such as has been reported here, the addition of this factor to the diet is without benefit. This is probably due to two separate causes, one being that the diet, while believed to be lacking in this vitamin, actually contains amounts sufficient to support normal growth; the other the effect of liver storage of vitamin B_{12}. It is entirely possible that rats weaned from mothers receiving adequate amounts of vitamin B_{12} are capable of storing sufficient quantities of this factor to carry them through a growth trial of 28 days' duration. Hartman, Dryden and Cary ('49) have reported that in order to produce a vitamin B_{12} deficiency in rats, it was necessary to place the mothers on a vitamin B_{12}-deficient diet during the lactation period. Therefore, in studies of this type one must know, not only the actual vitamin B_{12} content

of the test rations, but also the previous state of nutrition of the experimental subjects.

Metabolism studies

In order to determine more fully the true digestibility and efficiency of utilization by rats of rations containing amino acids as the nitrogen source, an experiment involving the use of the body balance technique was devised. The techniques involved have been described in detail by Swift, Kahlenberg, Voris and Forbes ('34). Twelve pairs of weanling, male, albino rats of the Wistar strain were used for this experiment. One member of each pair received the amino acid ration, while each litter mate received the same amount of food, nitrogen and energy from a similar ration containing intact casein as the source of nitrogen. The test ration used for this study contained 23.2% amino acids as the mixture AAM-VI. The composition of this amino acid mixture is given in table 1. The basic compositions of the two rations were the same as were given previously. Both rations were supplemented with the crude animal protein factor preparation [9] at the level of 0.5% of the ration. The amino acid ration was further supplemented with 1.05% corn oil, to make the two rations isocaloric.

The amino acid ration used in this experiment was one that had been found to give a relatively good rate of growth for rats. Although subsequent experiments indicated that further additions to this ration would improve the growth rate, it is believed that the amino acid ration used in this experiment supports a sufficiently good rate of growth of rats to make possible a satisfactory comparison of the utilization of free amino acids and intact casein.

The rats receiving the ration containing the free amino acids as the nitrogen source determined the food consumption of the pairs in the majority of cases. Specifically, of a total of 672 food offerings to the amino acid group, there were 440

[9] See footnote 8, page 248.

refusals. With the same number of food offerings to the group receiving the casein ration, there were only 64 refusals, with 51 of these from 4 rats. Figure 2 shows the comparative growth curves of the two groups. It can be seen from this

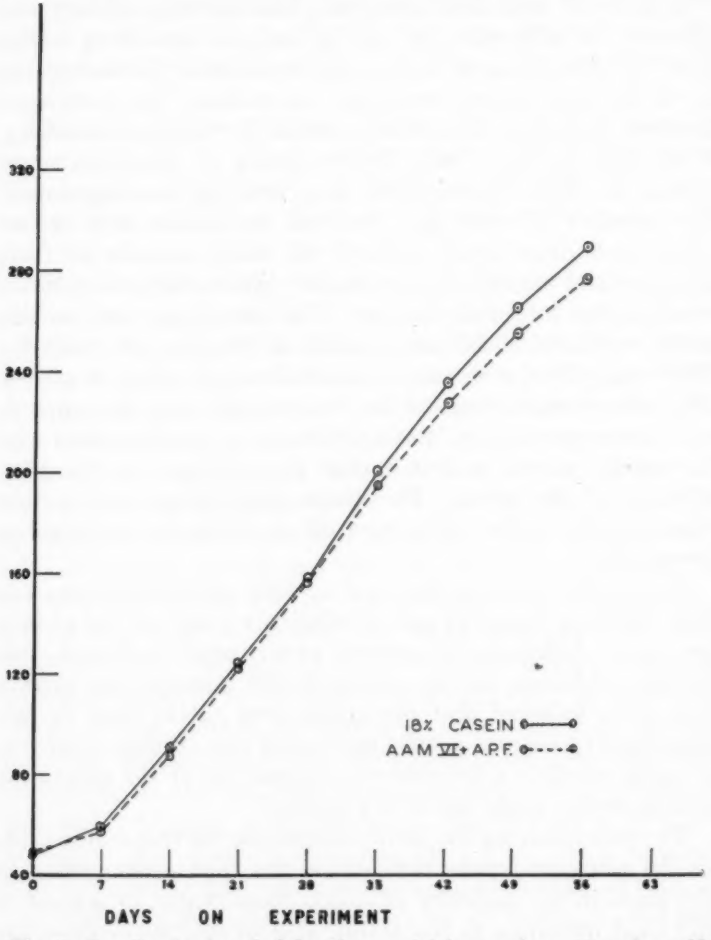

Fig. 2 Growth of rats pair-fed rations containing casein or 19 amino acids plus APF.

figure that the two groups grew at a comparable rate for the first 30 days, after which time the rats receiving the casein ration gradually increased their rate of gain over that of the rats receiving the amino acid ration. At the end of the experimental period of 56 days, the rats receiving the casein ration had reached an average weight of 290 gm, while the rats receiving the amino acid ration weighed only an average of 277 gm. Thus, even though the rats receiving the intact protein as the nitrogen source were limited in their food intake to the same levels as those received by the amino acid group,

TABLE 3

Average amounts of food eaten and gains in empty body weight, nitrogen and fat per rat during 56 days

SOURCE OF NITROGEN IN RATION	FOOD CONSUMED	EMPTY BODY WEIGHT [1]	
		Initial	Final
	gm	*gm*	*gm*
Free amino acids	688	42.6	265.2
Intact casein	688	42.3	278.6

SOURCE OF NITROGEN IN RATION	GAIN IN EMPTY BODY WEIGHT	NITROGEN OF BODY GAIN	FAT GAINED
	gm	*gm*	*gm*
Free amino acids	222.6	7.12	33.01
Intact casein	236.3	7.54	40.35

[1] Contents of alimentary tract removed.

they were still able to demonstrate a superior weight gain at the end of 56 days.

As was previously noted, the greatest difference in weight gain between rats receiving an amino acid ration and a control group receiving intact casein occurred during the first 7 days, after which time the two groups grew at a nearly equal rate. By introducing the paired feeding technique, such initial differences have been nullified and the superiority of the casein ration does not become apparent until after the 5th week.

Table 3 shows in summary form average values obtained for food consumption, gain in empty body weight, and gains

of nitrogen and fat for 56 days. The rats receiving the casein control ration showed significantly greater gains in empty body weight, nitrogen and fat than did the group receiving the amino acid ration. A statistical analysis of these differences, using the paired method of "Student," indicates that for all three values a highly significant difference exists between groups. The bodies of the casein control rats contained 17% fat, whereas the bodies in the other group contained only 15% fat. Furthermore, the fat constituted a greater percentage of the gain in the casein group than in the amino acid group.

A comparison of the distribution of ingested nitrogen is given in table 4. Since the rats receiving the intact casein

TABLE 4

Distribution of food nitrogen as affected by nitrogen source

	SOURCE OF NITROGEN IN DIET	
	Free amino acids	Intact casein
Food (gm)	18.04	18.04
Feces (gm)	0.63	0.95
Digested (gm) (Apparent)	17.41	17.09
% of food N	96.5	94.7
Urine (gm)	9.67	9.29
% of food N	53.6	51.5
Gain of N (gm)	7.12	7.54

ration excreted a significantly greater amount of nitrogen in the feces, the value for apparent digested nitrogen was significantly greater for the amino acid ration than for the control ration. It is to be noted that the digestion referred to here is apparent digestion, since no differentiation is made between total and metabolic nitrogen in the feces. However, since both groups of rats received the same levels of fiber, nitrogen and energy, and the body sizes were comparable, it is reasonable to assume that the losses of metabolic nitrogen were the same for the two groups.

No significant differences were noted between groups as to losses of urinary nitrogen. The body gain of nitrogen, how-

ever, was significantly greater for the control group than for the amino acid group. When calculated as per cent of ingested nitrogen, the group receiving the amino acids retained 39.5% of the ingested nitrogen, while the control rats retained 41.8%. This gain of body nitrogen was determined by direct analysis of the animal carcasses and not by the difference between ingested nitrogen and urinary and fecal losses. Although the apparent digestibility of nitrogen was higher for the amino acid ration, the retention of nitrogen from this ration was significantly less than that of rats fed a similar ration containing intact casein as the nitrogen source.

TABLE 5

Distribution of total food energy as affected by nitrogen source

	SOURCES OF NITROGEN IN DIET	
	Free amino acids	Intact casein
	Cal.	*Cal.*
Food	2,926	2,926
Feces	143	134
Digested	2,783	2,792
Urine	203	90
Metabolized	2,580	2,702
Body gain		
total	578	666
as protein	271	290
as fat	307	376
Heat production	2,002	2,036

The distribution of total food energy is given in summary form in table 5. The most striking difference between groups to be noted in this table is in urinary energy excretion. As can be seen, the rats receiving the free amino acid ration excreted more than twice the amount of energy in the urine than did the control group. Since the two groups lost the same amounts of nitrogen in the urine, as is shown in table 4, it may be assumed that the rats fed the ration containing only free amino acids as the source of nitrogen excreted the major portion of the nitrogen in the urine as amino acids rather than urea. Since the ratio of carbon to nitrogen is

greater in amino acids than in urea, the excretion of nitrogen in the form of amino acids would carry along with it a greater amount of energy. It is also possible that the D amino acids present in the amino acid ration are excreted as their corresponding keto analogues, and that the nitrogen is excreted in the urine as urea. Further work on this subject is necessary before a definite answer is possible.

Because of this much greater excretion of energy in the urine of the rats receiving the amino acid ration, the value for the metabolizable energy is significantly lower for the amino acid ration than for the intact casein ration. It has been observed by many workers that calorie utilization seems to be less efficient with diets containing amino acid mixtures, and such rations require higher calorie intakes than do similar rations containing casein as the nitrogen source. This may possibly be due to the higher excretion of energy in the urine of rats fed amino acid mixtures. As is clearly shown here, this excretion amounted to 6.9% of the total energy intake in the case of the amino acid ration, while only 3.1% of the energy intake was excreted in the urine by rats receiving intact casein. Also, since this amino acid mixture contained fewer calories per gram than did the same amount of nitrogen furnished in the form of intact casein, a higher amount of fat had to be added to the amino acid ration to make the two diets isocaloric. Furthermore, the results obtained here indicate that an even greater amount of energy must be furnished with the amino acid ration than with the casein ration, to give both groups of rats the same amounts of metabolizable energy.

A further study of the distribution of ingested energy shows that the casein control group retained significantly greater amounts of body gain of energy, both as protein and as fat. The heat production of both groups was of the same magnitude. However, if one relates the heat production to the metabolizable energy of the rations, a significant difference between groups is obtained. Such a comparison shows that 77.6% of the metabolizable energy of the amino

acid ration was converted to heat, whereas only 75% was converted to heat in the case of the control ration.

SUMMARY

Experiments have been conducted to compare the rate of growth of rats fed a casein ration with that of rats fed rations containing various mixtures of purified amino acids.

Apparently a period of adaptation to the amino acid diets was required by the rat, since during the first 7 days on experiment the rats receiving the optimum amino acid diet grew at a significantly slower rate than the control rats receiving the casein ration. After this adaptation period the two groups grew at nearly the same rate.

The addition of monosodium glutamate to an amino acid diet does not improve the palatability of the diet for the rat.

Vitamin B_{12} is necessary for optimum growth and food efficiency for rats, but an exogenous supply of this factor as an adjunct to all diets is not always required. This may be due to the presence of this factor in the diet, per se, as a contaminant; to sufficient storage of the vitamin in the livers of the test animals; or to its synthesis in the intestinal tract.

The addition of either streptomycin or aureomycin to the optimum amino acid diet produced a significant increase in the growth rate of rats. The resultant stimulation was comparable to that obtained by the addition of intact casein to the amino acid diet. Therefore growth factors such as strepogenin are not required, per se, in the diet of the rat.

The use of the body balance technique in conjunction with paired feeding has clearly shown, in the case of the rat, that the utilization of nitrogen and energy derived from free amino acids is less efficient than when the nitrogen is fed in the form of intact casein. This is shown by a lower average weight gain, less efficient utilization of the ration, decreased deposition of nitrogen and fat in the body, increased urinary excretion of energy, and a significantly lower metabolizable energy value for the amino acid diet.

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DIURNAL VARIATION IN THE EXCRETION OF CHROMIUM OXIDE AND LIGNIN [1]

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ONE FIGURE

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The ratio technique of determining digestibility offers the following advantages: It eliminates the necessity of making total collections; it permits digestion trials to be conducted in ordinary barn stalls; and it effects a saving of time, labor, and expense.

The accuracy of the digestibility values obtained by ratio techniques depends on the validity of the assumptions (1) that the inert (tracer) substances become evenly distributed with the ingesta, and (2) that they pass through the digestive tract in a manner similar to that of the ingested nutrients. If the inert substance and the nutrients were evenly distributed in the feces, then theoretically one fecal sample would be sufficient. If, on the other hand, the rate of excretion of the tracer material was inconstant, or if the nutrients in the ration were eliminated in an irregular manner, sampling errors would occur, and would affect the accuracy of digestibility values calculated by ratio techniques.

In a recent publication (Kane et al., '50), it was shown that digestibility coefficients obtained by the ratio technique did not differ significantly from those calculated by the total collection method when sampling errors were restricted by

[1] This work was done with funds made available by the Agricultural Research and Marketing Act of 1946.

taking all samples from the same source, the total collection aggregate. The sampling error, however, remained an unknown quantity.

The present paper concerns the sampling error. Digestibility coefficients obtained by ratio techniques, using chromium oxide and lignin as indicators, on 15 individual passages of feces ("grab" samples) were compared with digestibility coefficients obtained by the total collection method. In addition, the excretion rate of chromium oxide and lignin per passage was determined over a 24-hour period to define the amount of variation in chromium oxide and lignin excretion that occurred through a period of a day and a night.

EXPERIMENTAL PROCEDURE

To gain some idea of the size of the sampling error, samples of 15 individual passages of feces ("grab" samples) were taken from three cows on 5 different days during the time the cows were on digestion trials in a previously reported experiment (Kane et al., '50). The "grab" samples were collected in the afternoon between 2:00 and 4:00 P.M. The exact time of sampling was not recorded. The digestibility coefficients of the nutrients of the "grab" samples were calculated by the ratio technique, using both lignin and chromium oxide as inert materials, for comparison with digestibility figures obtained by the total collection procedure.

In order to devise an accurate sampling plan, it seemed that data on the normal variation in the rate of excretion of chromium oxide and lignin would be of value. Feces samples were taken individually from every passage for 24 hours from three animals that had just completed a 9-day digestion trial, data for which have been previously reported (Kane et al., '50). These cows had been receiving chromium oxide and lignin in the feed for over three months. The ration consisted approximately of 15 pounds of barn-cured alfalfa hay, 20 pounds of corn silage, and 8 pounds of grain. Each passage of feces was weighed and analyzed for dry matter, chromium oxide, and lignin. The analytical methods

used in these experiments were essentially the same as those used in the previous experiment (Kane et al., '50).

RESULTS AND DISCUSSION

The digestibility of the various nutrients of the "grab" samples as determined by ratio techniques in comparison with

TABLE 1

Comparison of digestibility coefficients obtained by grab samples and total collection

METHOD	DRY MATTER	PROTEIN	FIBER	NFE	ETHER EXTRACT
Period I — Alfalfa silage sample, April 6, 1948					
Total collection — 9 days	60.8	62.5	42.8	72.1	67.0
Chromium oxide [1]	66.2	70.0	51.8	72.4	68.6
Lignin [1]	60.6	65.0	43.9	71.9	63.3
Period I — Alfalfa silage sample, April 7, 1948					
Total collection — 9 days	60.8	62.5	42.8	72.1	67.0
Chromium oxide [1]	62.1	67.2	44.3	71.8	64.2
Lignin [1]	60.8	66.1	42.6	70.8	63.0
Period V — Barn-cured alfalfa sample, May 25, 1948					
Total collection — 9 days	63.6	69.8	45.7	74.1	51.7
Chromium oxide [1]	59.4	69.8	40.6	68.9	42.5
Lignin [1]	61.3	71.1	43.5	70.4	44.7
Period V — Barn-cured alfalfa sample, May 27, 1948					
Total collection — 9 days	63.6	69.8	45.7	74.1	51.7
Chromium oxide [1]	62.4	69.7	46.3	72.0	46.6
Lignin [1]	62.8	70.0	46.8	72.3	47.1
Period VI — Barn-cured alfalfa, June 8, 1948					
Total collection — 9 days	63.5	67.5	47.7	73.9	53.7
Chromium oxide [1]	63.5	68.0	50.5	73.2	49.5
Lignin [1]	61.9	66.6	48.4	73.2	47.0

[1] Single grab sample.

the values for the corresponding total collection periods are shown in table 1. Each figure reported represents an average for three cows. Analysis of the data for the digestibility values for the three sets of data (total collection, chromium oxide and lignin) revealed no significant difference in dry

matter or crude fiber. However, there were significant differences for protein and nitrogen-free extract at the 5% level, and highly significant differences for fat at the 1% level. It seems apparent that digestibility values obtained from single "grab" samples are not sufficiently accurate to be used in digestibility studies. It is noted, however, that in some cases, particularly in the case of a single "grab" sample of May 27, 1948, remarkably good agreement was obtained between the digestibility data of the two ratio techniques and the total collection method.

Figure 1 is a graphic illustration of variations found in the rates of excretion of chromium oxide and lignin in the animals on experiment for the 24 hours.

The chromium oxide and lignin means of the three animals for the 24-hour period agreed closely with similar averages for the 9-day digestion trial immediately preceding. The mean values for chromium oxide for the three cows were, respectively, 0.37%, 0.35% and 0.37% for the 24-hour period under study, and 0.38%, 0.35% and 0.36% for the 9-day digestion trial. The lignin means for the three cows were 23.4%, 22.8% and 22.5% for the 24-hour period, and 22.9%, 22.3% and 22.0% for the 9-day interval. The close agreement of these averages leads us to believe that no abnormal change had taken place in the excretion rate of our experimental animals in the period under study, and the data obtained in the 24 hours can be taken to represent a normal daily interval.

The results indicate that there is a diurnal variation in the excretion rate of chromium oxide and lignin by the dairy cow. The chromium oxide content of the feces of all three cows rose to the highest point around 9:00 A.M. Thereafter, the chromium oxide percentage decreased slowly until around 9:00 P.M. At that time, two of the three animals had sharp increases, which quickly subsided. From midnight on the chromium oxide content increased slowly until peaks were reached by all three cows around 9:00 A.M.

An analysis of variance on the chromium oxide content of the feces of the three cows for the 24-hour period reveals highly significant differences between A.M. and P.M. samples.

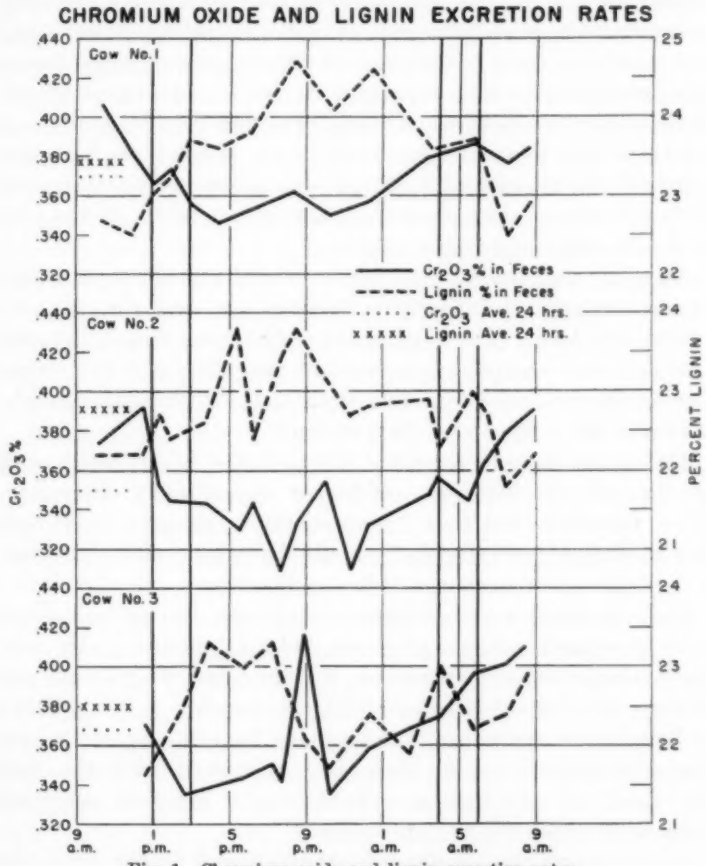

Fig. 1 Chromium oxide and lignin excretion rates.

This highly significant difference is attributed to a naturally occurring variation in the rate of chromium oxide excretion (fig. 1). This fluctuation indicates that the time of day samples are taken is important, and should be considered in de-

vising an accurate sampling plan for digestion studies using the ratio technique.

The lignin content of the feces displayed a different pattern from that of the chromium oxide. In figure 1, the lignin curve of cow no. 1 for rate of lignin excretions rose slowly and regularly from 8:00 A.M. to 8:00 P.M., then sloped downward gradually to its low point at 8:00 A.M. The lignin curves of the other two cows were more irregular than that of no. 1, but there was general agreement in the display of maximum values in the vicinity of 8:00 P.M. and minimum value around 8:00 A.M. These data show that diurnal variation in the rate of lignin elimination also occurs.

An analytical study of the variance of the lignin percentages showed significant differences between A.M. and P.M. samples at the 5% level. The significant differences found between A.M. and P.M. samples demonstrate that although the variation in lignin content is less than that of chromium oxide, it is still too large to be disregarded.

It is interesting to note that Edin et al. ('44), in their work on the use of chromium oxide for digestibility determinations, recommended that "representative samples from both day and night excrements" should be taken. Our data help to explain the reason for this specification.

Several authors have demonstrated that diurnal variations exist in regard to blood pressure, kidney function, pulse rate, body temperature, metabolism, and calcium, magnesium and citric acid content of the blood, to mention a few. There is little information as to why these fluctuations occur in a periodic 24-hour cycle. Are they connected with the food ingested? Or are they manifestations of internal biological laws with which we are unfamiliar?

If the variation of chromium oxide and lignin excretion were related to the time of ingestion of food, one would expect two maxima and two minima in the daily graph of a 24-hour cycle, since the experimental animals were fed twice a day (4:30 A.M. and 1:30 P.M.). Data reported above indicate that the variation is not associated with the time of

ingestion of food. While two of the three chromium oxide curves display double peaks, in one case the peaks (those at 9:00 P.M.) were very short in time and indefinite, in comparison to the strength and length displayed by the 9:00 A.M. peaks. In regard to the lignin data, only cow no. 3's curve gave a slight indication of two peaks.

In the literature, we note that Izzo ('49) has reported a diurnal variation of blood and urine glucose in diabetics. Izzo's urine glucose excretion curves are roughly similar to the chromium oxide curves displayed in figure 1, consisting of basic crests in the morning around 8:00 A.M. and lows at night. Izzo stated that all 15 patients studied manifested a diurnal rhythm of blood and urine sugar. He further claimed that this daily fluctuation was independent of changes in the diet; even starvation failed to eliminate it. In passing, it was observed that glucose, an essential carbohydrate metabolite, which becomes surplus and unusable in the disturbed human carbohydrate metabolism of the diabetic, displayed a daily pattern of excretion similar to that manifested in the bovine by chromium oxide, a surplus and indigestible inorganic metal oxide.

A possible explanation for the 12-hour difference found between the chromium oxide and lignin peak excretion rates may be due to the medium in which these two digestion indicators were fed. The chromium oxide was thoroughly mixed with the ground grain. The lignin was a natural constituent of the hay. According to Schalk and Amadon ('28), the major portion of ground grain is deposited in the anterior portion of the rumen. Due to its high specific gravity and the rapid absorption of water, the ground grain tends to sink and remain in the same location. Under these conditions, it would gain an early entrance into the reticulum and omasum. Hay, on the other hand, of light specific gravity and slow to absorb water, would be driven by the contractions of the reticulum to the posterior portions of the rumen, so that at least one complete circuit of the rumen would have to be made prior to regurgitation and remastication.

Data herein reported support one of the many conclusions of Schalk and Amadon ('28), who proposed that ingested food in the bovine had two alternate pathways within the rumen. According to these investigators, the morphology and physiology of the rumen enable a cow to separate the major portions of its food into two categories — concentrates and roughages. The concentrates, whose energy is readily available for metabolic purposes, remain in the anterior portion of the rumen and thus secure a rapid entry to the reticulum and omasum. On the other hand, roughages, whose energy is not readily available and therefore requires predigestion treatment by the rumen microorganisms, are forced to the rear of the rumen, from which point they must make a slow circuit of the rumen cavity prior to regurgitation and remastication. The well-known fact that cows maintained solely on concentrates cease to ruminate is in agreement with this theory.

It is possible that the interval between peak excretion rates of chromium oxide and lignin may be more than 12 hours; it could be 36, 60 or some higher value (n 24 + 12). The evidence provided by this paper is that a 12-hour lag occurs in the peak excretion rates between the two indicators. Schalk and Amadon ('28) estimate the average time food residues remain in the rumen as 12 or 24 hours. This estimate is in general agreement with the observations of Moore and Winter ('34) who, in a study of the rate of passage of inert materials through the bovine digestive tract, reported the peak excretion rate averaged 33 hours for iron oxide and 37 hours for rubber rings. Ewing and Wright ('18), in experiments based on digestion trials and subsequent slaughter, stated that the average time food residues remained in the rumen and reticulum was 61 hours. More work is evidently needed to clarify this disputed point.

Considerable variation was found in the dry matter content of the individual passages of all three cows. As the chromium oxide and lignin values have been expressed in percentages of the dry weight of the individual passages,

there was a possibility that the variation in dry matter content might limit the free expression of the chromium oxide and lignin variation due to the different bases used. Percentage values were transformed into actual weights of chromium oxide and lignin. Appropriate statistical treatment showed that error due to using percentage figures was insignificant.

The data accumulated in this experiment can be utilized in developing a more accurate system of fecal sampling in digestibility studies. On the assumption that cows in the future will display variations in chromium oxide and lignin excretion similar in character to those of the animals in this investigation, we have, with aid of figure 1, selected the period from 1:00 P.M. to 3:00 P.M. as the best time for daily sampling under the conditions of this experiment. It is recommended that all passages should be collected within this time interval, and if the cows do not defecate naturally in this period they should be made to do so. This time-interval was chosen because at that period both the chromium oxide and lignin percentages of the three cows approximated their averages for the 24-hour cycle. Another period that could be used for accurate sampling is the two-hour interval from 4:00 A.M. to 6:00 A.M. There is no reason why both these periods cannot be used and the number of samples doubled.

Another variant of this plan is the use of sampling periods equidistant from the time when average daily values of the indicators are being excreted. It has been found that chromium oxide excretion rates are above average in the morning and below average in the afternoon. Lignin is just the opposite. Hence, if periods of equal distance from the time when average value for the indicators is attained (2:00 P.M. under the conditions in our laboratory) are selected, the morning and evening variation errors should cancel, and a more accurate indicator excretion rate result. This plan has been tested at our station (unpublished data). In an experiment started prior to the time the conclusions and data of this paper became available, the periods of 10:00–

12:00 A.M. and 2:00–4:00 P.M. were designated as the sampling periods. Digestibility data obtained in a three-day trial by this method agreed satisfactorily with data secured in conventional 10-day trials, by total collection.

The practice of collecting and sampling most of the daily excreta has been found to yield satisfactory results for digestion studies using the chromium oxide technique (Andersen and Winther, '34). This practice, popular in Europe, apparently has been used with success. Naturally, as the amount of feces sample more closely approaches 100% for any 24-hour period, it will more closely approximate the true chromium oxide and nutrients averages, providing approximately equal amounts of day and night excreta are represented.

The method proposed essentially aims to avoid extreme and erroneous indicator excretion values. By confining the selection of sampling to hours when the diurnal variations of the indicators are minimum, indicator amounts recovered in excreta should correspond more accurately to the 24-hour average.

At this time, it is our belief that the use of the sampling plan recommended above will eliminate some of the error and lack of agreement present in digestion experiments performed by ratio techniques.

SUMMARY

1. Chromium oxide and lignin excretion rates of three experimental animals have been studied.

2. The previous finding that one fecal sample is inadequate for the determination of digestibility has been confirmed.

3. Significant diurnal variations in the rate of chromium oxide and lignin excretion occur in dairy cattle.

4. The character of the diurnal variation has been described.

5. An improved sampling plan for ratio technique digestibility determinations has been proposed.

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EFFECT OF ORAL ADMINISTRATION OF STREPTOMYCIN ON URINARY EXCRETION OF B VITAMINS IN MAN [1]

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The role of the intestinal flora in modifying the nutritional value of the diet has been studied in different ways (Elvehjem, '46; Melnick and Oser, '47). Changes in dietary carbohydrate have been shown to affect the growth of rats and the level of B vitamins found in the intestinal contents (Guerrant and Dutcher, '34; Morgan et al., '38; Mannering et al., '44). Addition of insoluble sulfonamides to certain diets results in poor growth, leukopenia and cytopenia in the rat, which can be corrected by administration of folic acid (Daft and Sebrell, '43). A decrease in the liver stores of biotin, folic acid and pantothenic acid, and in the levels of these vitamins in the feces, is also found in rats given sulfa drugs (Skeggs and Wright, '46). Under other conditions, addition of sulfa drugs or antibiotics to the diets of animals results in faster growth of the animals than is obtained with the diet alone (Moore et al., '46; Stokstad and Jukes, '50). Recent experiments have also shown that germ-free chicks grow and ap-

[1] A preliminary report was presented before the American Institute of Nutrition at Atlantic City, New Jersey, April, 1950 (*Fed. Proc., 9:* 369). This work was supported by grants from the Nutrition Foundation, Inc., the Williams-Waterman Fund of the Research Corporation, and the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service.

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parently develop as well as control chicks fed diets of known composition (Reyniers et al., '50).

In an attempt to determine whether changes in intestinal bacteria in man modify the level of B vitamins available on a given diet, the effect of oral administration of streptomycin on the urinary excretion of several B vitamins was studied in the present experiments. Previous studies in man have shown that the addition of phthalylsulfathiazole (sulfathalidine) to the diet had little effect on the urinary excretion of B vitamins, but led to a decreased fecal excretion of folic acid and biotin (Grundy et al., '47).

Other experiments reported here on the effect of streptomycin on the excretion of N^1-methylnicotinamide (N^1-Me) and the 6-pyridone of N^1-Me (pyridone) following supplements of tryptophan present further evidence that the conversion of tryptophan to nicotinic acid compounds takes place mainly in the body and not in the intestine.

EXPERIMENTAL

The subjects, with one exception (a staff member), were ward patients who were found to be free of complicating organic disease as determined by clinical and laboratory examinations, and were maintained in a separate metabolism ward.[3] They were closely supervised in regard to complete urine collection and strict adherence to their diets.[4] The diets differed for the various subjects and were carefully controlled. The caloric value and the content of thiamine, riboflavin, nicotinic acid and protein were calculated for each diet as shown in the tables. Two of the subjects received diets containing 70 or 100 gm of cornstarch per day, which was replaced by an equal weight of sucrose during part of the experiment. After suitable control periods streptomycin[5] (calcium chlo-

[3] Charity Hospital of Louisiana, New Orleans.

[4] Mor Beef (chopped beef) was kindly supplied for these diets by Wilson and Company, Inc., through the courtesy of Dr. E. J. Czarnetzky.

[5] Streptomycin (calcium chloride complex) was generously supplied by the Medical Division of Merck and Company, Inc.

ride complex) was given orally in capsules. Three of the subjects received 1 gm of streptomycin 4 times a day, and the other three subjects received this amount 6 times a day. For each gram of streptomycin, 1.45 gm of the calcium chloride complex was given.

Twenty-four-hour urines were collected in amber bottles containing 15 ml of glacial acetic acid, stored in the refrigerator and pooled in 48-hour periods for analysis. Riboflavin, nicotinic acid and tryptophan were measured by microbiological methods as previously modified (Sarett and Goldsmith, '49). After DL-tryptophan [6] supplements were administered, the ether-soluble tryptophan-like compound excreted in the urine was also measured (Sarett and Goldsmith, '49). Quinolinic acid was partially decarboxylated by autoclaving a sample of the urine in 1 *N* sulfuric acid solution and the resulting nicotinic acid was measured microbiologically (Sarett, '51b). The medium of Landy and Dicken ('42) was modified for assay of folic acid using *L. casei*. Free biotin was measured using *L. arabinosus* (Wright and Skeggs, '44) and total thiamine using *L. fermenti* (Sarett and Cheldelin, '44). In one experiment the urinary excretion of pyridoxine compounds was measured [7] with *S. carlsbergensis* (Atkin et al., '43). In all of the microbiological tests the values obtained using several levels of diluted urine agreed well and the test organisms did not appear to have been inhibited by any streptomycin present in the urine. Values for 4-pyridoxic acid were obtained fluorometrically but were discarded when it was shown that certain substances in the diet led to the excretion of interfering compounds (Sarett, '51a). N^1-methylnicotinamide (N^1-Me) and the 6-pyridone of N^1-Me (pyridone) were measured fluorometrically (Huff and Perlzweig, '47; Rosen et al., '49). Nitrogen values, obtained by the

[6] DL-tryptophan was generously furnished by the Medical Research Department, Winthrop-Stearns Chemical Company, Inc., through the courtesy of Mr. Kenneth Smoot.

[7] The pyridoxine analyses were kindly performed at Hoffmann-LaRoche, Inc., through the courtesy of Dr. Elmer DeRitter.

Kjeldahl method, and creatinine excretion, measured colorimetrically using an alkaline picrate solution, were determined in all samples to check the completeness of urine collection.

Feces were collected from each subject three times a week and weighed samples were serially diluted for bacterial counts. Incubation on MacConkey's agar was used for estimation of coliform organisms and non-lactose fermenting organisms. Blood agar was employed for detection of staphylococci and streptococci, and nutrient agar for total counts of many of the aerobic organisms. No attempt was made to estimate anaerobic organisms. These procedures were not designed to furnish a complete study of the bacterial flora of the stool, but were planned to provide some information on changes in bacterial flora, especially in regard to the coliform organisms.

RESULTS

In preliminary experiments, part of the carbohydrate content of the diets of subjects 1 and 2 was changed from starch to sucrose during the course of the experiments to see whether any change in B vitamin excretion would result (tables 1 and 2). The change of carbohydrate for these short periods appeared to have no effect on the urinary excretion of thiamine, riboflavin, and N^1-Me in either subject. The excretion of folic acid was decreased in subject 1 when the carbohydrate was changed from starch to sucrose, but no change was noted in subject 2. Administration of 4 gm of streptomycin per day to these subjects had little effect, if any, on the excretion of the above B vitamins (tables 1 and 2). Pyridoxine excretion was measured only in subject 2 and was not affected by changing part of the carbohydrate of the diet or by the streptomycin administration. The length of time the subjects were kept on the starch and sugar diets may have been too short, and the amount of streptomycin given too small, to obtain any definite changes in excretion.

Bacterial counts on the stool specimens showed no significant changes when the starch and sugar were interchanged

in the diets of subjects 1 and 2. In subject 2, streptomycin administration resulted in complete inhibition of coliform organisms within 4 days, which lasted for two days after the streptomycin was withdrawn. Occasional staphylococci were found during this time. In subject 1, bacterial counts on the stool showed little change from the control values until the

TABLE 1

Effect of diet and of oral administration of streptomycin on daily urinary excretion of B vitamins in subject 1 [1]

DIET [2]	DAYS	THIAMINE	RIBOFLAVIN	FOLIC ACID	N^1-ME
		μg	μg	μg	mg
Starch	1–6	200	800	7.0	2.3
Sucrose	7–18	300	750	3.5	3.0
Starch	19–26	250	560	3.6	2.9
Starch + 4 gm streptomycin per day	27–34	260	680	4.1	3.5
Starch	35–40	295	730	6.4	3.4

[1] Subject 1 (EM), male, age 42, weight 146 lb.

[2] Diet contained 100 gm starch or 100 gm sucrose: 2,800 cal., 84 gm protein, 1.4 mg thiamine, 2.6 mg riboflavin, 14 mg nicotinic acid.

TABLE 2

Effect of diet and of oral administration of streptomycin on daily urinary excretion of B vitamins in subject 2 [1]

DIET [2]	DAYS	THIAMINE	RIBOFLAVIN	FOLIC ACID	N^1-ME	PYRIDOXINE
		μg	μg	μg	mg	μg
Sucrose	1–8	165	1,250	2.9	6.7	84
Starch	9–14	170	1,380	2.9	7.6	77
Starch + 4 gm streptomycin per day	15–18	160	1,240	2.9	5.8	79
Sucrose + 4 gm streptomycin per day	19–24	160	1,460	3.3	5.9	72
Sucrose	25–30	155	1,300	3.0	4.7	66

[1] Subject 2 (FL) male, age 24, weight 148 lb.

[2] Diet contained 70 gm starch or 70 gm sucrose: 2,400 cal., 72 gm protein, 0.9 mg thiamine, 3.1 mg riboflavin, 15 mg nicotinic acid.

6th day of streptomycin administration, when no organisms could be detected by the methods used.

An experiment was set up in which two subjects were maintained on control diets for 16 days and then received 6 gm of streptomycin per day for 20 days (table 3). The excretion of thiamine, riboflavin, folic acid and quinolinic acid by subject 3 was not markedly affected by the streptomycin administration. A slight reduction in thiamine excretion and a more marked reduction in riboflavin excretion were observed in subject 4. In both subjects, the excretion of N^1-Me and pyridone was decreased slightly and the excretion of free biotin was decreased markedly. No coliform organisms and very few other organisms were found in the feces of subjects 3 and 4 from the third day of streptomycin administration to the end of the study. A few colonies of *Staphylococcus albus* were occasionally found during this period.

The effect of streptomycin administration on the excretion of nicotinic acid compounds following oral supplements of DL-tryptophan was studied in subjects 5 and 6 (table 4). After 4 days on the control diet subject 5 received 5 gm of DL-tryptophan per day for 6 days and the excretion of thiamine, riboflavin, folic acid, N^1-Me, pyridone, quinolinic acid, and tryptophan was measured. Four grams of streptomycin per day were then administered during a second period of 6 days on the control diet and 6 days with the tryptophan supplement. The increases in urinary excretion of quinolinic acid, N^1-Me, pyridone, and tryptophan, which followed administration of DL-tryptophan during the control period were similar to those found when streptomycin was added to the dietary regimen. The excretion of the ether-soluble tryptophan metabolite (as measured by the difference between the tryptophan values before and after ether extraction) was slightly higher during the period of streptomycin administration. The excretion of thiamine, riboflavin and folic acid increased throughout the experiment but did not appear to have been affected by the streptomycin administration. When the diet was supplemented with both tryptophan and streptomycin, there was

TABLE 3

Effect of oral administration of streptomycin on daily urinary excretion of B vitamins in subjects 3 and 4

DIET	DAYS	THIAMINE	RIBOFLAVIN	FOLIC ACID	BIOTIN	N^1-MR	PYRIDONE [3]	QUINOLINIC ACID
		μg	μg	μg	μg	mg	mg	mg
Subject 3 [1]								
Control	1–8	615	970	2.8	35	7.0	15	5.6
	9–16	625	835	2.7	37	6.6	14	5.5
Control + 6 gm streptomycin per day	17–26	585	880	2.5	24	6.3	13	5.5
	27–36	600	780	2.7	18	5.8	12	5.5
Subject 4 [2]								
Control	1–8	1,200	1,510	3.5	37	7.0	23	6.2
	9–16	960	1,400	3.6	33	7.4	..	6.3
Control + 6 gm streptomycin per day	17–26	890	1,150	4.0	25	5.2	22	5.5
	27–36	780	1,060	4.6	24	5.0	17	7.2

[1] Subject 3 (AF) female, age 49, weight 153 lb. Control diet: 1,750 cal., 58 gm protein, 1.1 mg thiamine, 1.5 mg riboflavin, 11 mg nicotinic acid.

[2] Subject 4 (ML) male, age 41, weight 167 lb. Control diet: 2,700 cal., 95 gm protein, 1.6 mg thiamine, 2.5 mg riboflavin, 17 mg nicotinic acid.

[3] Pyridone analyses available for only 4 days of each period.

TABLE 4

Effect of oral administration of streptomycin on daily urinary excretion of B vitamins and on conversion of tryptophan to nicotinic acid compounds in subjects 5 and 6

DIET		DAYS	THIAMINE	RIBOFLAVIN	FOLIC ACID	BIOTIN	N^1-ME	PYRIDONE	QUINOLINIC ACID	TRYPTOPHAN	
										As is	After ether extraction
			μg	μg	μg	μg	mg	mg	mg	mg	mg
Subject 5 [1]											
Control		1–4	160	470	2.1		1.9	7	4.0	19	18
Control + 5 gm DL-tryptophan per day		5–10	240	850	2.4		8.3	31	13.1	82	53
Control	+ 4 gm streptomycin per day	13–18	215	970	2.6		2.3	8	4.2	19	18
Control + 5 gm DL-tryptophan per day	+ 4 gm streptomycin per day	19–24	300	1,090	5.8		7.3	39	13.9	112	68
Subject 6 [2]											
Control		1–10	230	860	2.1	32	2.9	5	5.3	11	10
Control + 5 gm DL-tryptophan per day		11–22	260	740	1.9	35	9.5	24	8.7	88	40
Control	+ 6 gm streptomycin per day	23–32	280	890	1.8	26	4.6	8	4.4	10	10
Control + 5 gm DL-tryptophan per day	+ 6 gm streptomycin per day	33–44	240	640	1.8	17	10.2	27	10.5	85	35

[1] Subject 5 (LS), female, age 39, weight 122 lb. Control diet: 2,100 cal., 70 gm protein, 1.1 mg thiamine, 2.1 mg riboflavin, 10 mg nicotinic acid.

[2] Subject 6 (SD), female, age 57, weight 108 lb. Control diet: 1,600 cal., 55 gm protein, 0.9 mg thiamine, 1.5 mg riboflavin, 9 mg nicotinic acid.

an increased excretion of thiamine and folic acid which may be related to changes in intestinal flora. The coliform organisms in the feces of this subject decreased in number during streptomycin administration, but some persisted throughout the experiment.

A similar experiment was carried out in subject 6, allowing longer periods for control and for supplementation and increasing the streptomycin to 6 gm per day. The increase in excretion of nicotinic acid metabolites by this subject following tryptophan administration was not affected by the streptomycin administration (as also shown for subject 5) and lends support to the evidence that the conversion of tryptophan to nicotinic acid compounds takes place mainly in the body (Henderson and Hankes, '49; Hurt et al., '49; Snyderman et al., '49) and not in the intestine (Ellinger and Abdel Kader, '49). Thiamine, riboflavin and folic acid excretion were not affected by the streptomycin or tryptophan administration, while biotin excretion was decreased when streptomycin was given. In fecal cultures obtained during the period of streptomycin administration, no coliform organisms and only a few colonies of *Streptococcus viridans* were found.

DISCUSSION

Measurement of the urinary excretion of B vitamins (or their metabolites) during a period when the metabolic activity of the bacteria of the intestines is altered by streptomycin may give some idea as to whether these bacteria usually play an important role in modifying or supplementing the dietary intake of B vitamins. In the present experiments, administration of 6 gm of streptomycin per day was generally accompanied by a marked decrease in biotin excretion, and a small decrease in excretion of nicotinic acid metabolites (N^1-Me and pyridone) within 10 days. This suggests that the bacterial flora may normally furnish to the body a relatively large amount of biotin (Oppel, '42) and a small amount of nicotinic acid above that provided by the diet. This nicotinic acid does not appear to be derived from dietary

tryptophan, since adddition of streptomycin to the diet has no effect upon the excretion of nicotinic acid compounds following tryptophan supplements. Most of the evidence suggests that the main conversion of tryptophan to nicotinic acid takes place within the body (Henderson and Hankes, '49; Hurt et al., '49; Snyderman et al., '49).

In most of the subjects tested the excretion of thiamine and riboflavin was not markedly influenced by the streptomycin administration, and in the one subject tested pyridoxine excretion was also not affected. The excretion of folic acid was not decreased in any of the subjects when streptomycin was given. The occasional finding of an increase in excretion of folic acid and other B vitamins when streptomycin is administered may be related to the growth-promoting action of some of the antibiotics (Moore et al., '46; Stokstad and Jukes, '50).

The differences in growth and vitamin synthesis which can be produced in animals by changing the carbohydrate of the diet, or by addition of sulfonamides or antibiotics to the diet, suggest that intestinal bacteria can modify the level of some of the B vitamins available in the diet. It seems likely that some of the bacteria may be synthesizing B vitamins (available to the host), while other bacteria may be destroying or utilizing them. When this equilibrium is upset by one of the techniques mentioned above, conditions may permit either the synthetic or the destructive flora to predominate.

SUMMARY

The effects of oral administration of streptomycin on the urinary excretion of thiamine, riboflavin, biotin, folic acid, pyridoxine, N^1-methylnicotinamide (N^1-Me) and the 6-pyridone of N^1-Me (pyridone), and on the excretion of nicotinic acid compounds following tryptophan supplements, were studied in 6 subjects maintained for 24 to 44 days on regulated diets. After suitable control periods, streptomycin (calcium chloride complex) was administered orally for 8 to 22 days in doses of 1 gm given either 4 or 6 times daily. Within a

few days, fecal cultures showed either complete or partial inhibition of growth of coliform organisms and almost complete absence of the other aerobic organisms studied.

The excretion of folic acid was not decreased during streptomycin administration and under certain conditions appeared to be increased. Thiamine and riboflavin excretion were decreased in one subject and not markedly affected in the others. Excretion of pyridoxine compounds was determined in one subject and found to be unaffected by streptomycin. Biotin excretion was markedly decreased during streptomycin administration, and excretion of N^1-Me and pyridone appeared to be slightly reduced.

The addition of DL-tryptophan to the control diet led to increases in excretion of quinolinic acid, N^1-Me and pyridone. This conversion of tryptophan to nicotinic acid compounds did not appear to be affected by streptomycin administration and the concomitant change in intestinal flora. This supports the evidence that the main conversion of tryptophan to nicotinic acid takes place in the body, and not in the intestine.

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INCREASED SURVIVAL OF LIVER-FED RATS ADMINISTERED MULTIPLE SUBLETHAL DOSES OF X-IRRADIATION [1]

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Considerable data are available indicating that in addition to the known nutrients, substances are present in our diet which may be required in increased amounts during conditions of stress. Such factors are apparently dispensible under normal conditions, or the requirements for them are so small they may readily be met by amounts present in the diet or through the synthetic activity of the intestinal flora or the animals' own tissues. Certain drugs or other "stress factors" may, however, increase requirements for these substances to such an extent that deficiencies occur, manifested by retarded growth or tissue pathology, and preventable by the administration in appropriate amounts of the missing nutrient (Ershoff, '48a, '51a). Whole liver is a potent source of such unknown nutrients. Thus whole liver, or fractions thereof, has been shown to counteract the deleterious effects of massive doses of strychnine (Battelli, '40), sulfanilamide (Chamelin and Funk, '43), promin (Higgins, '44; Ershoff and McWilliams, '49), atabrine (Ershoff, '48b, '50a) and dinitrophenol (Ershoff, '50b). Similar results have been ob-

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served following the administration of toxic doses of diethylstilbestrol (Chamelin and Funk, '43), alpha-estradiol (Ershoff and McWilliams, '48a), desiccated thyroid (Ershoff, '47a, b; Betheil et al., '47), thyroxin, thyroglobulin and iodinated casein (Ershoff and McWilliams, '48b) and cortisone acetate (Ershoff, '52). Liver has also been found to contain a factor distinct from any of the known B vitamins which significantly increased the capacity of rats to swim in cold water (Ershoff, '51b). In the present communication data are presented on the comparative effects of liver and the known B vitamins on survival after x-irradiation in the rat.

PROCEDURE AND RESULTS

The basal ration employed in the present experiment consisted of sucrose, 61%; casein,[2] 24%; salt mixture,[3] 5%; cottonseed oil,[4] 10%; and the following synthetic vitamins per kilogram of diet: thiamine hydrochloride, 20 mg; riboflavin, 20 mg; pyridoxine hydrochloride, 20 mg; calcium pantothenate, 60 mg; nicotinic acid, 60 mg; ascorbic acid, 200 mg; 2-methyl-naphthoquinone, 10 mg; and choline chloride, 2 gm. To each kilogram of diet were also added 4,000 U.S.P. units of vitamin A[5] and 400 U.S.P. units of vitamin D.[6] The vitamins were added in place of an equal amount of sucrose. Each rat also received once weekly a supplement of 4.5 mg alpha-tocopherol acetate. In addition to the basal ration the following diets were also employed: (1) basal ration plus the following additional vitamin supplements per kilogram of diet: thiamine hydrochloride, 20 mg; riboflavin, 20 mg; pyridoxine hydrochloride, 20 mg; calcium pantothenate, 60 mg; nicotinic acid, 60 mg; biotin, 5 mg; folic acid, 10 mg; *p*-aminobenzoic acid, 400 mg; inositol, 800 mg; and vitamin B_{12}, 150 μg;

[2] Vitamin-test casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

[3] Hubbel, Mendel and Wakeman salt mixture, General Biochemicals, Inc., Chagrin Falls, Ohio.

[4] Wesson.

[5] MYVA-DRY Powder, Distillation Products, Rochester, New York.

[6] HY-DEE Powder, Standard Brands, New York.

and (2) basal ration plus 10% whole liver powder.[7] The vitamin supplements and the whole liver powder were added in place of an equal amount of sucrose.

All experiments were performed with female rats of the Long-Evans strain, 23 to 25 days of age at the start of the experiment. Animals were kept in metal cages with raised screen bottoms to minimize access to feces and were fed the above diets ad libitum. Diets were made up weekly and stored under refrigeration. Rats were fed on alternate days. All food not consumed 48 hours after feeding was discarded. These measures were employed to minimize oxidative changes in the diet.

The radiation factors were as follows: GE Model Maximar 250; 250 kv; 15 ma; 0.5 mm Cu and 1 mm Al filters plus a Cu parabolic filter;[8] HVL, 2.15 mm Cu; target distance to top of box, 78 cm; and dose rate, 17.92 r per minute (measured in air). The animals to be irradiated were placed in a wooden box divided into 14 compartments 7 cm wide, 16 cm long and 10 cm deep (10 rats were irradiated at a time). The partitions and top were made of 1/8″ cellulose acetate sheeting; and the top, one side and bottom of each compartment were perforated with holes for purposes of ventilation. The container was rotated slowly on an electrically-driven turntable to insure equivalent irradiation.

Experiment No. 1. Comparative effects of whole liver powder and the known B vitamins on the survival of adult rats administered multiple sublethal doses of x-irradiation

Thirty female rats were employed in the present experiment. Animals were selected at an average weight of 45.6 gm

[7] Desiccated liver, Armour and Co., Chicago, Illinois.

[8] A non-uniform filter which produces a flat isodose surface of x-ray intensity constructed by the method of Greenfield and Hand ('52). We are indebted to Dr. Moses Greenfield and Miss Katherine Hand of the Atomic Energy Project, University of California at Los Angeles, for the construction of the parabolic filter. The center of the filter had a thickness of 1.7 mm Cu; the edge, 0.5 mm Cu.

(range, 41 to 53 gm) and were fed ad libitum the three diets indicated above (10 rats per group). After 22 weeks of feeding the average body weights of rats in the three groups were as follows: basal ration, 235.5 gm; basal ration plus B vitamins, 258.6 gm; and basal ration plus liver, 250.9 gm. At this time the rats were administered a single exposure of 200 r x-irradiation, which was repeated at weekly intervals until a total of 1,600 r (8 exposures) had been administered. Animals were continued on their respective diets until 30 days after the last x-ray exposure. Results are summarized in table 1.

TABLE 1

Comparative effects of whole liver powder and the known B vitamins on the survival of adult rats administered multiple sublethal doses of x-irradiation (10 animals per group)

DIETARY GROUP	INITIAL BODY WT.	SURVIVING [1]	AVERAGE SURVIVAL TIME OF DECEDENTS [2,3]
	gm	*%*	*days*
Basal ration	235.5	40	35.5 ± 7.7
Basal ration + B vitamins	258.6	20	41.1 ± 4.9
Basal ration + whole liver	250.9	80	61.0 ± 1.0

[1] Experimental period, 79 days. X-irradiation was administered over a period of 49 days. The experiment was terminated 30 days after the last x-ray exposure.

[2] Based on length of survival after first x irradiation.

[3] Including standard error of the mean calculated as follows: $\sqrt{\frac{\Sigma d^2}{n}} / \sqrt{n}$, where "d" is the deviation from the mean and "n" is the number of observations.

A significant difference was observed between the various groups in the survival of rats administered multiple sublethal doses of x-irradiation. Sixty per cent of the rats fed the basal ration and 70% of the group fed the basal ration plus B vitamin diet died before the first rat fed the basal ration plus whole liver diet succumbed. Thirty days after the last x-ray exposure 80% of the rats fed the latter diet were still alive, in contrast to a mortality rate of 60% and 80%, respectively, for animals on the basal ration and basal ration plus B vitamins. No significant change in body weight or

gross appearance was observed in the various groups as a result of x-ray administration. Surviving animals in the various groups gained an average of 9 to 21 gm during the experimental period.

Experiment No. 2. Comparative effects of whole liver powder and the known B vitamins on the survival of adult rats administered a single lethal dose of x-irradiation

One hundred and twenty female rats were employed in the present experiment. Animals were selected at an average weight of 44.6 gm (range, 39 to 52 gm) and were fed ad libitum the three diets indicated above (40 rats per group). After 16 weeks of feeding the average body weights of rats in the three groups were as follows: basal ration, 227.7 gm; basal ration plus B vitamins, 238.3 gm; and basal ration plus liver, 235.6 gm. At this time 20 rats in each group were administered a single dose of 800 r x-irradiation; the remaining half in each group received 850 r. Animals were continued on their respective diets for 30 days post-irradiation.

No significant difference was observed between the various groups in the per cent or length of survival of rats administered a single lethal dose of x-irradiation. In the series receiving 800 r the mortality rate for the various groups was as follows: basal ration, 60%; basal ration plus B vitamins, 60%; and basal ration plus whole liver, 55%. In the series receiving 850 r, the mortality rate was: basal ration, 90%; basal ration plus B vitamins, 70%; and basal ration plus whole liver, 80%. Surviving animals in the various groups lost an average of approximately 10% body weight during the 30-day period following x-irradiation. The average survival time of decedents in the various groups ranged from 7.1 to 10.3 days.

Experiment No. 3. Comparative effects of whole liver powder and the known B vitamins on the survival of immature rats administered multiple sublethal doses of x-irradiation

Sixty female rats were employed in the present experiment. Animals were selected at an average weight of 45.4 gm (range, 38 to 53 gm) and were fed ad libitum the three diets previously described (20 rats per group). On the 4th day of feeding 10 rats in each group were administered a single exposure of 200 r x-irradiation, which was repeated at weekly intervals until a total of 1,600 r (8 exposures) had been administered. The remaining 10 animals in each group served as untreated controls. Rats were fed their respective diets for 200 days or until death, whichever occurred sooner. Results are summarized in table 2.

The findings are in agreement with those reported in experiment 1. A significant difference was observed between the various dietary groups in the survival of rats administered multiple sublethal doses of x-irradiation, the average survival time of irradiated rats fed the liver-containing ration being approximately twice that of animals fed the basal ration or basal ration plus B vitamin diet. Gain in body weight was retarded in all irradiated rats. Growth retardation was particularly marked after the 4th week of radiation, and was somewhat greater on the basal ration and basal ration plus B vitamin diet than on the liver-containing ration. Graying of the fur and "aging" was noted in a number of irradiated rats by the 12th week of feeding. It occurred, however, to an equal degree on all three rations. Two of the irradiated rats fed the basal ration developed marked paralysis and extensive edema of the paws a week or so before their death. These findings were not observed in remaining animals in this group or in irradiated rats fed the other diets. No significant difference in growth or gross appearance was observed in non-irradiated rats on the various rations.

DISCUSSION

The findings of the present experiment indicate that the average survival time of rats administered multiple sublethal doses of x-irradiation was significantly greater on a puri-

TABLE 2

Comparative effects of whole liver powder and the known B vitamins on growth and survival of immature rats administered multiple sublethal doses of x-irradiation (10 animals per group)[1]

DIETARY GROUP	INITIAL BODY WT.	GAIN IN BODY WEIGHT ON FOLLOWING DAYS OF EXPT.			PER CENT SURVIVING [2]	AVERAGE SURVIVAL TIME OF IRRADIATED RATS [3,4]
		32nd	60th	100th		
	gm	*gm*	*gm*	*gm*		*days*
			X-ray series			
Basal ration	45.8	90 (10)	99 (5)	123 (2)	0	68.7 ± 8.7
Basal ration + B vitamins	46.2	99 (10)	83 (4)	104 (1)	10	66.4 ± 10.2
Basal ration + whole liver	45.0	99 (10)	116 (8)	151 (5)	30	117.2 ± 12.9
			Control series			
Basal ration	43.8	127 (10)	156 (10)	186 (10)	100	..
Basal ration + B vitamins	43.5	129 (10)	164 (10)	188 (10)	100	..
Basal ration + whole liver	42.0	139 (10)	176 (10)	203 (10)	100	..

[1] The values in parentheses indicate the number of animals which survived and on which averages are based.

[2] Experimental period, 200 days.

[3] Based on length of survival after first x-irradiation. Averages were computed on the basis of a 196-day survival time for animals alive at the termination of the experiment.

[4] Including the standard error of the mean. See footnote 3, table 1.

fied diet containing 10% whole liver powder than on a similar ration in which the B vitamins were provided in synthetic form. Similar results were obtained both with adult and weanling rats. The longer survival of rats fed liver was

apparently not due to its content of any of the known B vitamins. This is indicated by the fact that supplements of all the known B vitamins were without significant effect on the per cent or length of survival of multiply-irradiated rats. Available data do not indicate, however, whether the protective effect of liver was due to an unidentified factor or factors or to its content of other known nutrients. Since liver also contains a factor (distinct from any of the known B vitamins) which counteracted the toxic effects of thyroid (Ershoff, '47a, b, '50c; Betheil et al., '47), promin (Ershoff and McWilliams, '49) and atabrine (Ershoff, '48b, '50a) administration, further work is indicated to determine what relationship, if any, exists between the "antitoxic factor of liver" (Ershoff, '51a) and the factor or factors indicated above.

Present findings indicate that although liver exerted a protective effect on rats receiving multiple sublethal doses of x-irradiation, it was without significant effect on the survival of rats receiving a single lethal dose. These findings suggest that liver was protecting the irradiated rat not from radiation injury per se but possibly from noxious agents which may have been formed or accumulated in toxic amounts following multiple sublethal irradiation.

SUMMARY

Experiments were conducted on the effects of (1) multiple sublethal doses of x-irradiation and (2) a single lethal dose of x-irradiation on the survival of rats fed: (a) a purified basal ration, (b) the basal ration plus supplements of all the known B vitamins, and (c) the basal ration plus 10% whole liver powder. The average survival time of both immature and adult female rats following multiple sublethal doses of x-irradiation was significantly greater on the liver-containing ration than on the other diets tested. The protective factor in liver was distinct from any of the known B vitamins. No significant difference in per cent or length of survival was observed among the three dietary groups following a single lethal dose of x-irradiation.

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A STUDY OF THE RELATIONSHIP OF LIVER XANTHINE OXIDASE TO QUALITY OF DIETARY PROTEIN[1]

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THREE FIGURES

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It has been demonstrated that dietary amino acids greatly influence liver xanthine oxidase activity (Williams et al., '49; Williams and Elvehjem, '49, '50; Litwack et al., '50). The rapid loss of this enzyme activity induced by a non-protein regimen proceeds at a greater rate than the loss of the flavin adenine dinucleotide coenzyme or other liver proteins (Litwack et al., '50). Since liver xanthine oxidase activity has been shown to be very sensitive to amino acid availability in dietary proteins (Williams and Elvehjem, '49) and to deficiencies of essential amino acids, such as methionine (Williams et al., '49) and tryptophan (Williams and Elvehjem, '50), one would infer that a correlation between the activity of the enzyme and the quality of the dietary protein might be demonstrated. A study of this relationship between liver xanthine oxidase activity and the proteins casein, lactalbumin, gliadin and meat has been made and is presented here, with a method for evaluating the value of dietary protein in terms of liver xanthine oxidase activity of the animals fed the protein.

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EXPERIMENTAL

In these studies adult male Sprague-Dawley strain rats were allowed a 7-day adjustment period on a good stock ration before being placed on the experimental diets. All of the experimental rations were designed in the same way and were varied only in the type of protein. The basal ration consisted of the following components: protein in levels of 5, 10, 18 and 30%; vitamin mixture (Williams and Elvehjem, '49a), 2%; salts IV (Hegsted et al., '41), 4%; corn oil, 5%; and sucrose for the balance to give a total of 100%.

Each animal was supplemented orally with two drops of halibut liver oil per week. Food and water were given ad libitum and the animals were housed individually in separate cages. A total of 440 animals was used in the enzyme studies. Protein nitrogen was determined for the samples of casein, lactalbumin, gliadin and meat protein used in these studies with the semi-micro Kjeldahl technique. For these samples, the following values were obtained: casein,[2] 13.4% nitrogen; lactalbumin,[3] 12.6% nitrogen; gliadin,[4] 15.6% nitrogen; and meat protein preparation 9.4% nitrogen. The meat protein preparation consisted of beef round, which was ground and dried at 50°C. in drying ovens. The dried product was further ground in a Wiley mill to a fine powder and used in this form. Casein was fed at 5, 10, 18 and 30% levels, with each variation compensated for by the level of carbohydrate. Each protein used was fed at the same level of casein based on its nitrogen content, so that more protein was added to the ration when the protein was low in nitrogen compared to casein and vice versa.

Xanthine oxidase activity was determined in the livers of 4 to 6 animals fed each of the protein levels after one, three, 5, 7, and 9 or 10 days of feeding. Each complete experiment with each of the proteins was repeated two or three times,

[2] General Biochemicals, Ohio.
[3] Borden Co., New York, N. Y.
[4] Huron Milling Co., Michigan.

and the results are an average of all of these experiments. The animals were stunned by a blow on the head, decapitated and exsanguinated. The livers were removed and placed immediately in cracked ice. After being chilled for several minutes, the livers were blotted free of moisture, weighed, and homogenized (Potter and Elvehjem, '36) in 5 volumes of ice-cold 0.039 M sodium-potassium phosphate buffer (pH 7.4); the xanthine oxidase activity was then determined in a Warburg respirometer, as described by Axelrod and Elvehjem ('41), at 30°C. Liver nitrogen was determined in aliquots of the homogenates, using a micro-Kjeldahl technique. The enzyme activity was calculated in terms of fresh liver weight and liver nitrogen.

In order to observe whether a correlation between the enzyme data and the criterion of growth exists, the same feeding experiments were repeated using weanling Sprague-Dawley strain male rats. The growth data were recorded every three days for a period of two weeks and results were calculated as change in weight in grams per week per rat. Four weanling rats were used at each level of the various proteins for the growth determinations. This involved a total of 48 weanling rats in the growth experiments.

RESULTS AND DISCUSSION

Procedure for evaluating dietary protein in terms of liver xanthine oxidase

In assessing the results, a method was sought whereby the mass of collected data could be condensed into a suitable quantitative form. This was accomplished by plotting the level of dietary protein as the abscissa, and enzyme activity (expressed as $\mu L O_2$ per hour per gram fresh liver or per gram liver nitrogen) as the ordinate for a specific day in the feeding trial, as shown in figure 1 for casein. Each dietary protein gave a similar set of curves. The slopes of the curves were then calculated by the least squares method for curve-

fitting and the average line possessing such a slope was drawn through the points. By plotting the slopes thus obtained versus the day on which they were obtained, it was found with the proteins employed in these experiments that a leveling-off began after the 5th day, as is shown in figure 2. In other words, the slopes of the curves relating xanthine oxidase activity to the level of dietary protein began to approach

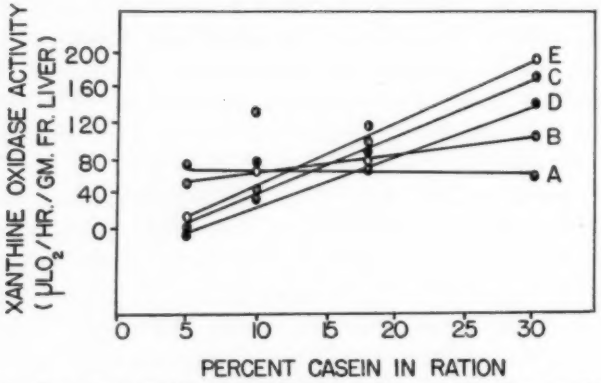

Fig. 1 The effect of the level of dietary casein upon the liver xanthine oxidase activity of rats after different intervals of time. Each point represents the average of three separate experiments, in which 4 rats were used for each point in each experiment. The best straight line has been drawn after calculating the equation for each line by the method of least squares.

Curve A = ◒ = day 1
Curve B = ◑ = day 3
Curve C = ◑ = day 5
Curve D = ● = day 7
Curve E = ○ = day 10

a maximum after the 5th day and leveled off by the 9th or 10th day. Calculation of the data in terms of enzyme activity based on liver nitrogen gave essentially the same results as with enzyme activity based on liver fresh weight.

Since the rate of change of xanthine oxidase activity as related to the concentration of dietary protein leveled off by the 9th or 10th day, it appeared that by that time the animals were in equilibrium with their respective dietary regi-

mens. Although the gliadin curve began to level off earlier and fell slightly by the 9th day, the relative change was so slight that it might be considered negligible. Therefore, in evaluating a protein in terms of maintenance of liver xanthine oxidase activity, the different levels of the protein need be fed only for a 9- or 10-day period, omitting the enzyme studies on days one, three, 5 and 7. With the experimental data

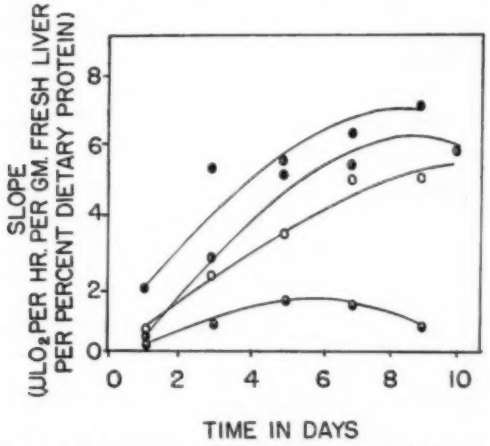

Fig. 2 The slope of the lines obtained in figure 1 plotted versus the day at which that slope obtained. Each curve in this figure was obtained from a set of curves similar to the set shown in figure 1 for each dietary protein.

● = beef round
◑ = casein
○ = lactalbumin
◒ = gliadin

for the 9th day, a curve obtained in a manner similar to that employed in obtaining the 9th day curve in figure 1 will possess a slope relating liver xanthine oxidase activity to the dietary protein being studied.

From figure 2 it can be seen that on the 9th day the efficiencies of the proteins in maintaining liver xanthine oxidase, calculated as μLO_2 per hour per gram fresh liver per per cent of dietary protein, were as follows: meat protein,

+ 7.0; casein, + 5.5; lactalbumin, + 5.0; and gliadin, + 0.6. It can be seen from the terms of the slope that these values relate liver xanthine oxidase activity to dietary protein much more exactly than if only a single level of the dietary protein is employed. Of the proteins studied, meat protein is therefore most effective in maintaining liver xanthine oxidase activity. Casein and lactalbumin give a good and almost

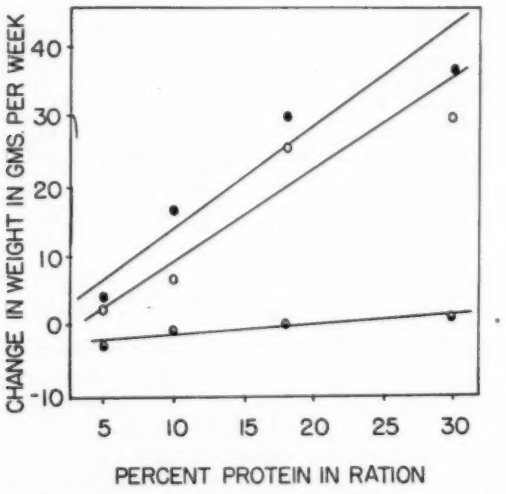

Fig. 3 A comparison of the growth-promoting ability of three dietary proteins used in these experiments. The change in weight, in grams, of weanling rats per week is plotted versus the level of protein in the ration.

● = lactalbumin
○ = casein
◒ = gliadin

equal response, while gliadin gives a poor response, having an efficiency of nearly zero.

The growth data for weanling rats using the same proteins as in the enzyme experiments are shown in figure 3. The level of protein is plotted as the abscissa against the change in weight in grams per week per rat, plotted as the ordinate. The data have been subjected to the same

type of analysis as the enzyme data of figure 1. Beef round was not included in the growth experiments because not enough of the same sample used in the enzyme experiments was available. Therefore it was considered best to omit the beef round from the growth experiments, since another sample prepared some months after the first would probably have different properties from that used for the enzyme studies. The other proteins, casein, lactalbumin and gliadin, however, were taken from the same batches as those used in the enzyme experiments. From figure 3 it can be observed that the slope for the casein curve is 8.8 times that of gliadin; and the slope for lactalbumin, 9.5 times that of gliadin. If one compares these values with the enzyme data, it can be seen that the slope for casein is 9.2 times that for gliadin and the slope for lactalbumin is 8.3 times that of gliadin, so that the enzyme data in general correlate well with the growth data except that the casein and lactalbumin figures are reversed. The latter protein promotes growth slightly more than it excites the enzyme response. It is likely that the enzyme maintenance measurements involve protein metabolism more specifically than does the growth criterion, since xanthine oxidase itself is a protein. These studies are being continued and expanded to elucidate further the relationship between dietary protein and tissue enzyme activity.

SUMMARY

1. The interrelationships between the quality of dietary protein and the maintenance of liver xanthine oxidase activity in rats have been investigated.

2. A quantitative method for evaluating the quality of dietary protein in terms of liver xanthine oxidase activity has been derived from these studies. Using this method for evaluating various proteins, the following relative values were obtained, assuming gliadin equals 1.0: meat protein 11.7; casein 9.7; lactalbumin 8.3; and gliadin 1.0.

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